SATURABLE DISTRIBUTION OF TACRINE INTO THE STRIATAL EXTRACELLULAR FLUID OF THE RAT: EVIDENCE OF INVOLVEMENT OF MULTIPLE ORGANIC CATION TRANSPORTERS IN THE TRANSPORT

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The kinetics and mechanism by which tacrine is distributed in the rat brain were examined. Tacrine levels in plasma and striatal extracellular fluid were used to evaluate the pharmacokinetics of this process. The $K_{D,brain}$ was decreased with the dose for tacrine, indicating that the distribution to the brain is saturable. The uptake of organic cations such as choline, 1-methyl-4-phenylpyridinium (MPP), tetraethylammonium (TEA), and carnitine was inhibited by the addition of tacrine to cultures of mouse immortalized brain capillary endothelial cells. In addition, the apical to basal transport and basal to apical transport of tacrine were inhibited by the addition of organic cations to cultures of LLC-PK1 cells, suggesting that tacrine transport across the blood-brain barrier (BBB) is mediated by organic cation transport system(s). Consistent with the in vitro results, a standard reverse transcription-polymerase chain reaction procedure was able to amplify the message of mOCT2 and mOCTN2, but not mOCT1, in MBEC4 (mouse brain microvessel endothelial cell line 4) cells. Similarly, mRNAs for rOCT2 and rOCTN2 were present in representative rat brain samples. To determine whether OCT2 and/or OCTN2 transport tacrine, these transporters were cloned and then transfected in SK-HEP1 and HEK 293 cells. The uptake of choline, MPP, and TEA was inhibited by the presence of tacrine in rOCT2-expressing SK-HEP1 cells, whereas the uptake of carnitine was inhibited by the presence of tacrine in rOCTN2-expressing HEK 293 cells. Collectively, these observations suggest that the transport of tacrine across the BBB is mediated, at least in part, by multiple organic cation transport systems in rats.

Cholinesterase inhibitors, such as tacrine (Fig. 1A) and donepezil, are clinically useful in the treatment of Alzheimer’s disease (Summers et al., 1986; Dooley and Lamb, 2000; Jann et al., 2002). Although these drugs are believed to be pharmacologically active as the result of their ability to stabilize neurotransmitters in the brain (Ogura et al., 2000; Jann et al., 2002), alternate mechanisms, e.g., the inhibition of β-amyloid precursor protein secretion, may participate in the enhancement of memory in patients with Alzheimer’s disease (Lahiri, 1994). Since the site of action for the cholinesterase inhibitors would be the brain, an understanding of the kinetic process(es) involved in the brain distribution would be essential for therapeutic optimization. Unfortunately, however, only limited literature information is available regarding the neuropharmacokinetics of these cholinesterase inhibitors. For example, in a study involving the administration of radiolabeled tacrine to the rat, the radioactivity associated with the brain was found to be at least comparable to or higher than that found in the plasma (McNally et al., 1989), suggesting that carrier-mediated transport system(s) may be involved in the penetration of tacrine across the BBB. However, since radioactivity does not necessarily represent intact tacrine levels in the plasma and the brain, a precise estimation of the brain permeability of tacrine was not possible. A brain microdialysis method has also been used to estimate the brain permeability of tacrine in rats (Telting-Diaz and Lunte, 1993). In this previous study, the authors reported that the area under the concentration-time curve for the hippocampal ECF is approximately 80% of that for plasma after intravenous tacrine administration (1 mg/kg), indicating that tacrine is not readily permeable to the brain. However, the in vivo recovery of tacrine was not adequately evaluated in the study and, as a result, the brain permeability data reported may have been inaccurate. Furthermore, Brundage (1996) reported that the in vivo recovery of tacrine was highly variable, which complicates the estimate of permeability. Currently, the kinetics of tacrine distribution to the brain has not been fully characterized. Furthermore, the kinetics of its distribution into the brain has not been reported in the literature for cases of other cholinesterase inhibitors.

The objective of this study was to investigate the kinetics of the brain distribution of tacrine, a model cholinesterase inhibitor, and to elucidate the mechanism involved in brain transport. We were partic-
ularly interested in the potential involvement of carrier-mediated transport in the distribution of the model cholinesterase inhibitor to the brain, since it appears that the concentration of tacrine in the brain is apparently higher than that in the plasma. Our findings show that the kinetics for tacrine penetration across the rat BBB are saturable and that the transport is mediated, at least in part, by the organic cation transport system(s).

Materials and Methods

Materials. Tacrine (1,2,3,4-tetrahydro-9-aminoacridine; Fig. 1A), 9-aminoacridine (Fig. 1B), 9-aminoacridanone (Fig. 1C), choline, MPP, TEA, and cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO). [14C]Mannitol (specific activity 57 mCi/mmol) was purchased from Amer Sham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK), and [3H]mannitol (specific activity 22.3 Ci/mmol) were obtained from American Radiolabeled Chemicals (St. Louis, MO). [3H]Choline (specific activity 81 Ci/mmol), [3H]MPP (specific activity 70 Ci/mmol), [14C]TEA (specific activity 2.4 mCi/mmol), and [14C]mannitol (specific activity 22.3 Ci/mmol) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Keta- mine (Ketalar; Yuhan Co., Seoul, Korea, Korean Pharmacopoeia, 7th edition) and acipimazone (Sedaject, Samu Chemical Co., Seoul, Korea, Pharmcopeia, 7th edition) were also used in this study. The primers used in the RT-PCR study were synthesized by Takara Shuzo Co. (Shiga, Japan). ExTaq DNA polymerase, restriction enzymes, T4 ligase, and DH5α competent cells were provided from Takara Shuzo Co. or Promega (Madison, WI). Solvents were of HPLC grade, and all other chemicals were of analytical grade.

Animals. Male Sprague-Dawley rats (Dae-Hin Biocell, Eumsung, Korea), weighing 270 to 310 g, were used in all in vivo experiments. Experimental protocols involving the animals used in this study were reviewed by the Animal Care and Use Committee of the College of Pharmacy, Seoul National University, according to the National Institutes of Health guidelines (National Institutes of Health publication number 85-23, revised 1985) in the Guide for the Care and Use of Laboratory Animals. At all times except for the surgical procedure, the rat had free access to food and water, including during the microdialysis sampling period.

Surgical Procedure for Microdialysis Study. Rats were anesthetized by intramuscular administration of 20 mg/kg ketamine and 10 mg/kg acepromazine. After confirmation of the absence of a reflex reaction from a toe pinch test, the rat was mounted on a stereotaxic instrument (Stoelting Co., Wood Dale, IL) for the implantation of a guide cannula (MD-2201; BAS Bioanalytical Systems Inc., West Lafayette, IN) in the striatum region of the rat. A midsagittal incision was made to expose the skull, and the guide cannula, fitted with a dummy probe, was implanted into the striatum with the coordinates of 2.6 mm lateral, 0.2 mm anterior, and 6.5 mm ventral to the bregma (Paxinos and Watson, 1986). After the insertion, the cannulae were anchored to two screws with dental cement, and the rat was allowed to return to the individual cage for recovery. One week after the implantation, the animal was anesthetized again, and the femoral artery and vein were catheterized with PE50 tubes (BD Biosciences Discovery Labware, Bedford, MA). The polyethylene tubes were then externalized to the back of the rat, and a syringe was filled with a heparin solution (i.e., 40 units of heparin per ml of saline) and connected to the cannulae to prevent blood clotting. The dummy probe was removed from the guide cannula and the microdialysis probe, presoaked in 5 μM 9-aminoacridine (Fig. 1B), was carefully introduced through the cannula. The perfusion of the probe with dialysate medium was then initiated.

When it was necessary to determine the integrity of the BBB after the 7-day recovery period, [3H]mannitol (6 μCi/rat) was injected into the femoral vein, and the radioactivity in the plasma and dialysate was determined by liquid scintillation counting (Wallac 1409; PerkinElmer Life and Analytical Sciences, Boston, MA).

In Vitro Probe Recovery. An MD-2200 2-mm microdialysis probe (BAS Bioanalytical Systems Inc.), presoaked in 5 μM 9-aminoacridine (Fig. 1B), was placed in 2 ml of saline solution containing 250 nM tacrine at 37°C. The probe was perfused with a solution containing a retrocalibrator (1 μM 9-aminoacridanone; Clement et al., 1998) at a flow rate of 2 μl/min using a Harvard-22 syringe pump (Harvard Apparatus Inc., Holliston, MA). The concentrations of tacrine and 9-aminoacridanone were then determined by HPLC (see below). The recovery of tacrine and the loss of 9-aminoacridanone in vitro were determined using the following equations.

\[
\text{Recovery of tacrine} = \frac{C_{\text{dialysate}}}{C_{\text{medium}}} (1)
\]

\[
\text{Loss of 9-aminoacridanone} = \frac{C_{\text{perfusate}} - C_{\text{dialysate}}}{C_{\text{perfusate}}} (2)
\]

In Vivo Probe Recovery. Using a separate group of rats, the loss of tacrine and 9-aminoacridanone from the dialysate to the striatal ECF was determined. In this method, in vivo recovery was estimated by the assumption that the movement of the drug across the dialysis probe is essentially identical regardless of the direction. Stereotoxic surgery, cannulae implantation, and animal recovery were carried out in a manner similar to that described in the previous section. Upon insertion, the microdialysis probe was perfused at a flow rate of 2 μl/min using saline containing 250 nM tacrine and 1 μM 9-aminoacridanone. The perfusate leaving the probe was collected at 20-min intervals up to 6 h, and the concentration of tacrine and 9-aminoacridanone in the perfusate was determined by HPLC. The retrodialysis loss of tacrine and 9-aminoacridanone in vivo was calculated using eq. 3.

\[
\text{LOSS}_{\text{in vivo}} = \frac{C_{\text{perfusate}} - C_{\text{dialysate}}}{C_{\text{perfusate}}} (3)
\]

Pharmacokinetic Study. Tacrine (1 mg/kg, 2 mg/kg, or 5 mg/kg dose) was administered to the rats (administration volume 1 ml/kg in all dose levels) in the form of a bolus via the intravenous cannula. The dialysis medium that equilibrates with the striatal ECF was continuously collected at 20-min intervals up to 6 h. Blood samples (150 μl) were collected from the catheter connected to the artery at predetermined time points and centrifuged to collect plasma. The plasma and dialysate samples were frozen at −20°C until used for analysis. The concentration of tacrine in the ECF was calculated as follows:

\[
C_{\text{ECF, tacrine}} = \frac{C_{\text{tacrine, dialysate}}}{\text{Recovery}_{\text{tacrine}}} = \frac{C_{\text{tacrine, dialysate}}}{\text{LOSS}_{\text{tacrine, in vivo}} \times F} (4)
\]

\[
F = \frac{\text{LOSS}_{9\text{-aminoacridanone, in vivo}}}{\text{LOSS}_{\text{tacrine, in vivo}}} (5)
\]

Determination of Tacrine by HPLC. Tacrine concentrations in the plasma were determined by HPLC with a Shimadzu RF-10A fluorescence detector (Shimadzu, Kyoto, Japan) using 9-aminoacridanone (Fig. 1C) as an internal standard. Fifty microliters of Tris buffer (pH 10) was added to an aliquot (50 μl) of plasma sample, and tacrine was extracted by the mixed solvent (1 ml)
containing ethyl acetate and cyclohexane (1:1 by volume). The mixture was centrifuged at 6400 rpm for 5 min, and the organic layer was transferred and evaporated to dryness. Residue was reconstituted with 100 μl of mobile phase (see below), and a 20-μl aliquot was injected onto the HPLC system. For the determination of tacrine in the dialysate and the cell culture media, an aliquot was directly injected onto the HPLC system without any processing of the sample. In this application, an internal standard was not added. Peak height was used as an assay parameter in this study by using a Hitachi D-7500 integrator (Hitachi Ltd., Tokyo, Japan). The separation was performed on a reversed-phase HPLC column (4.6 × 150 mm C-18 column; GL Science Inc., Tokyo, Japan), and analytes were eluted with a mixture of triethylamine, filtered double distilled water, and methanol (5:450:550 by volume, pH adjusted to 4.5). The separation was carried out using a flow rate of 1.3 ml/min. Fluorescence in the eluent was monitored at an excitation wavelength of 330 nm and an emission wavelength of 365 nm. Calibration curves for the plasma, dialysate, and cell culture medium were constructed in the tacrine concentration range of 5 to 5000 nM. The retention times for tacrine and 9-aminocarboxybutanol (internal standard or the retrocalibrator) were 3.5 and 1.5 min, respectively. The response of the detector was linear in the concentration range examined (i.e., 5–5000 nM), and inter- and intraday variations in the assay were less than 15%, indicating that the assay is valid in the concentration range of interest.

Uptake of Organic Cations in MBEC4 Cells. MBEC4 cells, an immortalized mouse brain microvessel endothelial cell line 4, were used as an in vitro model of the BBB. Cells were maintained at 37°C in Dulbecco’s modified Eagle’s medium (low glucose) containing 10% fetal bovine serum and antibiotics under an atmosphere of 5% CO2 and 90% humidity (Hommen et al., 1999). MBEC4 cells (1 × 10⁶) were seeded in 12-well plates (Costar 3402; Corning Glassworks, Corning, NY), and the uptake experiment was performed 2 days after seeding. Uptake (3 min) of choline, MPP, and TEA (for all substrates, 100 nM) was studied at 37°C in the absence or presence (0.01, 0.1, 1, 10, or 100 μM final concentration) of tacrine. Carnitine uptake was also examined in the presence of 0.1, 1, 10, 100, or 1000 μM tacrine concentration. After aspiration of the medium, the incubated cells were washed three times with 1.5 ml of ice-cold phosphate-buffered saline. The radioactivity in the cells was determined after lysing the cells with 0.4 ml of 0.2 N NaOH.

Identification of mRNA for Organic Cation Transport Systems in MBEC4 Cells and the Rat Brain. In the literature, a number of transport systems for organic cations in the body have been proposed. In this study, we were primarily interested in the identification of the OCT1, OCT2, and OCTN2 in MBEC4 cells and the rat brain. To determine the presence of mRNA for mOCT1, mOCT2, and mOCTN2 in MBEC4 cells, RT-PCR was carried out using specific primers for these murine transporters. MBEC4 cells were harvested (1 × 10⁶ cells) and total RNA was extracted using an RNAeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). The RNA LA PCR Kit Ver.1.1 (Takara Shuzo Co.) was used for the reverse transcription. Reverse transcription was performed under the following conditions: 42°C for 10 min, 99°C for 5 min, and 5°C for 5 min. The synthesized first strand cDNA was used for PCR using a set of primers (Table 1). The PCR reaction mixture (40 μl) contained primers (0.2 μM), deoxynucleoside-5′-triphosphate (0.2 μM), ExTaq DNA polymerase (2 units), and the buffer provided. Standard PCR amplification was performed using the GeneAmp PCR system 2400 (Applied Biosystems, Foster, CA).

To amplify the mRNAs for rOCT2 and rOCTN2 in the rat brain, a commercially available rat brain cDNA (BD Biosciences Clontech, Palo Alto, CA) was used. In this study, standard PCR procedure was carried out using the specific primers and thermocycling conditions given in Table 1. Other experimental conditions were similar to the PCR procedure for MBEC4 cells.

Transport of Tacrine in the Presence of Organic Cations in LLC-PK1 Cell Monolayers. LLC-PK1 cells (ATCC number CL-101; passage 200-210) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 4 mM L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in an atmosphere of 5% CO2 and 90% relative humidity (Li et al., 2002). For the transport experiments, the LLC-PK1 cells were grown on a permeable polycarbonate insert (Costar 3401; Corning Glassworks) in 12 Transwell plates, and medium was changed at 2-day intervals. Transport was studied 5 days after seeding.

In the literature, the expression of organic cation transport systems is polarized in epithelial cells (Saito, 1997). Therefore, depending on the type of organic cation transport system, the direction of transport was examined differently (e.g., for the inhibition study with choline, MPP, and TEA, basal to apical transport; for the inhibition study with carnitine, apical to basal transport). For measurement of the basal to apical transport of tacrine, 1.5 ml of transport medium containing tacrine (1 μM final concentration) was added to the basal side of the Transwell in the presence or absence of choline, MPP, or TEA (for all potential inhibitors, the concentration was fixed at 500 μM). An aliquot (400 μl) of the medium in the apical compartment was collected at 10-min intervals and the tacrine concentration was determined by HPLC. For apical to basal transport, 0.5 ml of transport medium containing tacrine (1 μM final concentration) was added to the apical side in the presence of carnitine (500 μM final concentration). An aliquot (400 μl) of the medium in the receiver compartment was collected at 10-min intervals, and the tacrine concentration was determined by HPLC. The transport rate of tacrine was compared in the presence and absence of organic cations.

Cloning of rOCT2 and the Functional Expression in SK-HEP1. To determine whether rOCT2 (GenBank/European Molecular Biology Laboratory accession number D83484) is involved in the transport of tacrine, the transporter was cloned and stably transfected to SK-HEP1 cells (ATCC number HTB-52). rOCT2 was cloned from a rat kidney primary mRNA (BD Biosciences Clontech), and the SMART RACE procedure (BD Biosciences Clontech) was applied to reverse transcription. The full-length rOCT2 cDNA was obtained by PCR using rOCT2-specific primers: 5′-CCCCAACAGTTCCGCCGC-3′ (sense strand; underlined region for HindIII site) and 5′-CGCTTCAGGTGATGGAGTGTTGGTGAATG-3′ (antisense strand; underlined region denotes the XhoI site). The PCR reaction mixture (40 μl) contained primers (0.2 μM), deoxynucleoside-5′-triphosphate (0.2 μM), ExTaq DNA polymerase (2 units), and the buffer provided. Thermocycling consisted of 30 cycles of 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C on GeneAmp PCR system 2400. The amplification product (1.8 kb) was cloned into pcDNA 3.1/myc-His (Invitrogen, Carlsbad, CA), and the identity of the insert was confirmed by sequencing. The cloned cDNA contained the complete coding region (92–1873 of the rOCT2 cDNA) and was under the control of the T7 promoter in the plasmid. The rOCT2 was transfected to SK-HEP1 cells in six-well plates. After washing with serum-free medium, each culture dish was

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Sense Strand</th>
<th>Antisense Strand</th>
<th>Annealing Temperature</th>
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<tbody>
<tr>
<td>mOCT1</td>
<td>5′-ACGATGTTCTGAGACACCTTT-3′ (base 51–70)</td>
<td>5′-AGCCCAAATTTCAACACAGAT-3′ (base 1031–1050)</td>
<td>54–58°C</td>
</tr>
<tr>
<td>mOCT2</td>
<td>5′-TGCGACACCTTTGATATTTT-3′ (base 11–30)</td>
<td>5′-ACGACACAGTAAAAAACC-3′ (base 2031–2050)</td>
<td>56°C</td>
</tr>
<tr>
<td>mOCTN2</td>
<td>5′-AGCTGTCTGAAATGAGGGA-3′ (base 101–120)</td>
<td>5′-TTCCAGGCCTACTCTCTTT-3′ (base 528–547)</td>
<td>56°C</td>
</tr>
<tr>
<td>rOCT2</td>
<td>5′-CCCCAACAGTTCCGCCGACCCACGACCAC-3′ (base 92–110)</td>
<td>5′-CCGCCGGTACCTTTTCTCCTGTAACAGG-3′ (base 1853–1873)</td>
<td>58°C</td>
</tr>
<tr>
<td>rOCTN2</td>
<td>5′-CCCCAACAGTTCCGCCGACCCACGACCAC-3′ (base 92–110)</td>
<td>5′-CCGCCGGTACCTTTTCTCCTGTAACAGG-3′ (base 1776–1797)</td>
<td>56°C</td>
</tr>
</tbody>
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incubated at 37°C for 4 h with 2 ml of serum-free medium, which contained 10 μl of Lipofectamine 2000 (Invitrogen) and 4 μg of a plasmid DNA of pcDNA3.1-OCT2. The transfection medium was subsequently replaced by the standard culture medium. Transfected SK-HEP1 cells were selected by incubating the cells in a medium containing 1 mg/ml G418 (Invitrogen) for 3 weeks. The expression of rOCT2 was verified by RT-PCR at the end of the selection period and the function examined by MPP uptake (Okuda et al., 1996).

**Uptake of Organic Cations in rOCT2-Transfected SK-HEP1 Cells.** SK-HEP1 cells were maintained at 37°C in Dulbecco’s modified Eagle’s medium (low glucose) containing 10% fetal bovine serum under an atmosphere of 5% CO2 and 90% humidity. Cells (5 x 10⁴) were seeded in a 12-well plate (Corning Glassworks) and the uptake experiment was performed 2 days after seeding. Cells were incubated with a 2 μM concentration of choline, MPP, and TEA for 3 min at 37°C in the absence or presence (50, 100, 500, 1000, 5000, or 10,000 nM final concentration) of tacrine. After aspiration of the medium, the incubated cells were washed three times with 1.5 ml of ice-cold phosphate-buffered saline. The radioactivity in the cells was determined after lysing the cells with 0.4 ml of 0.2 N NaOH. The rate of uptake of organic cations was compared in the presence and absence of tacrine.

**Cloning and Transfection of rOCT2 and Uptake of Carnitine in rOCT2-Transfected HEK 293 Cells.** rOCT2 (GenBank/European Molecular Biology Laboratory access number AF104161) was cloned from a rat kidney primary mRNA (BD Biosciences Clontech), and standard reverse transcription was performed. The full-length OCT2 cDNA was obtained by PCR using rOCT2-specific primers: 5’-CCCCAACTCATGCTCCACCAT-GCgggACTACAgGAgF-3’ (sense strand; underlined region for HindIII site) and 5’-CCgggTACCTTTTCACCTTTGACAggG-3’ (antisense strand; underlined region for KpnI site). The amplification product (1.7 kb) was cloned into pcDNA3.1/CT-GFP (Invitrogen) and the cloned cDNA was transfected to HEK 293 cells. Cells were cultured at 37°C in Dulbecco’s modified Eagle’s medium (low glucose) containing 10% fetal bovine serum under an atmosphere of 5% CO2 and 90% humidity. Cells (2 x 10⁴) were seeded in 12-well plates, and 2 μg of cloned pcDNA3.1-OCT2 was transfected with 4 μl of Lipofectamine 2000. The uptake experiment was performed 24 to 30 h after transfection. Cells were incubated with a 1 μM concentration of carnitine for 4 min at 37°C in the absence or presence (0.1, 1, 10, 100, or 1000 μM final concentration) of tacrine. After aspiration of the medium, the incubated cells were washed three times with 1.5 ml of ice-cold phosphate-buffered saline. The radioactivity in the cells was determined after lysing the cells with 0.4 ml of 0.2 N NaOH. The rate of uptake of carnitine was compared in the presence and absence of tacrine.

**Data Analysis.** To determine the elimination clearance (CL) and the steady-state volume of distribution (Vss) for tacrine, a moment analysis was carried out. The area under the concentration curve in the plasma versus time from time 0 to infinity (AUC0-∞) and the area under the respective first moment time curve from time 0 to infinity (AUMC0-∞) were calculated using linear trapezoidal and area extrapolation methods (Gibaldi and Perrier, 1982). Equations 6 and 7 were then used to calculate the clearance and the volume for tacrine.

\[
\text{CL}_{\text{elimination}} = \frac{\text{Dose}}{\text{AUC}_{0-\infty}} \tag{6}\]

\[
V_{\text{ss}} = \frac{\text{AUMC}_{0-\infty}}{\text{AUC}_{0-\infty}} \times \text{CL}_{\text{elimination}} \tag{7}\]

In the potential inhibition study, the IC50 values were calculated from inhibition plots based on eq. 8, using nonlinear regression analysis.

\[
y = \frac{V_o}{1 + \left(\frac{C_{\text{tacrine}}}{IC_{50}}\right)^n} \tag{8}\]

In this equation, \(y\) and \(V_o\) are the uptake of cations in the presence and absence of tacrine, \(C_{\text{tacrine}}\) is the concentration of tacrine as an inhibitor, and \(n\) is the Hill coefficient.

When it was necessary to compare means between the treatments, a one-way ANOVA, followed by Duncan’s test, was typically used. For a comparison of the inhibitory effect of organic cation on tacrine transport in the LLC-PK1 cells, the Student’s t test was used to compare the means. \(p < 0.05\) was taken as denoting statistical significance. Data are expressed as the mean ± standard deviation (S.D.).

**Results**

**In Vitro Recovery of Tacrine and Loss of 9-Aminoacridanone across the Microdialysis Probe.** Based on an examination of the chemical structure of tacrine, 9-aminoacridanone (Fig. 1C) was selected as a potential retrocalibrator for the microdialysis study. However, in an in vitro recovery study suggested that the temporal profile of 9-aminoacridanone loss from the dialysate to the medium appeared to be significantly different from that for the recovery of tacrine and that the equilibration for tacrine and the retrocalibrator may be mediated by a slow process (data not shown). Therefore, we examined the possibility of enhancing the equilibration by pretreating the probe with 9-aminoacridine (Fig. 1B), an organic cation and a chemical analog of tacrine. After pretreatment with 9-aminoacridine, the temporal profiles for the loss of 9-aminoacridanone and the recovery of tacrine across the probe were almost superimposable (data not shown). In addition, the temporal profiles reached a plateau at the first collection period (i.e., 0–20 min of dialysis). These observations indicated that pretreatment appears to have eliminated the limiting binding problem and that the rate of diffusion of tacrine and 9-aminoacridanone was accelerated. As a result, 9-aminoacridanone appeared to be a reasonable retrocalibrator for tacrine after the above pretreatment. Subsequently, the in vivo recovery for tacrine was further studied.

**Loss of Tacrine and 9-Aminoacridanone in Vivo.** The in vivo loss of tacrine and 9-aminoacridanone was compared to determine the feasibility of 9-aminoacridanone as an in vivo retrocalibrator for tacrine. The temporal profiles for the loss of tacrine and 9-aminoacridanone were nearly identical after the 9-aminoacridine pretreatment and independent of the collection time after 30 min (data not shown). Since the diffusion of tacrine is likely to be identical regardless of the direction of drug movement across the probe (Zhao et al., 1995), the calculated \(F\) value would be expected to be similar to that obtained for the in vivo recovery of tacrine and loss of the retrocalibrator (Bouw and Hammarlund-Udenaes, 1998). The mean \(F\) value of 0.79 was used throughout the study to calculate the concentration of tacrine in the ECF from the dialysate concentration.

**Pharmacokinetics of Tacrine in the Plasma and the Striatal ECF of the Rat.** Temporal profiles of tacrine levels in the plasma are shown in Fig. 2A. In general, the plasma concentration declined in a multipexponential manner. Based on a moment analysis, the systemic clearance was found to be independent of the dose administered in the range of 1 to 5 mg/kg (Table 2). The steady-state volume of distribution decreased slightly with dose, although a one-way ANOVA failed to detect a statistical difference (Table 1; e.g., 12.5 ± 3.28 l/kg for 1 mg/kg dose versus 8.43 ± 1.72 l/kg for 5 mg/kg). The large volume of distribution may be partly derived from the significant protein binding of the drug, as evidenced by the fact that the free fraction of tacrine was small (Makela et al., 1994).

In the literature, the spatial dependence on different regions of the brain has not been reported for the expression of transporters (e.g., organic cation transport system). Since neuropharmacokinetics via microdialysis sampling is frequently studied in the striatum region (e.g., Xie and Hammarlund-Udenaes, 1998), we selected this region to examine the neuropharmacokinetics of the drug. The brain ECF concentration reached a plateau at 50 min postdose, and the profile declined in an exponential fashion (Fig. 2B). The ECF concentration
after 2 mg/kg administration was not statistically different from that after the administration of 1 mg/kg (Table 2). In addition, the peak ECF concentration after the administration of 5 mg/kg was approximately 1.07-fold higher than the peak ECF concentration at 1 mg/kg, indicating that the ECF concentration is not proportional to the dose and that a nonlinear process may be involved in the BBB penetration of the drug. Consistent with this, the distribution coefficient (K_D,brain; viz., AUCbrain divided by AUCplasma) for tacrine from the plasma to the ECF was decreased with dose (Table 2; p < 0.05 one-way ANOVA), indicating that tacrine penetration across the BBB is mediated by saturable kinetics.

**Uptake of Organic Cations in MBEC4 Monolayers.** Since tacrine is an organic cation, the saturable distribution of the drug into the brain may be mediated by organic cation systems. To examine this possibility, the transport of tacrine across the MBEC4 cells, an in vitro model of the BBB, was examined. In a preliminary study, the concentration dependence of the apical to basal transport for apically added tacrine was examined in the MBEC4 cells grown on a polycarbonate membrane. However, because of the significant leakage of solutes across the MBEC4 cell monolayer, coupled with a limited assay sensitivity, neither tacrine transport nor an uptake study for tacrine in MBEC4 was possible. Instead, the inhibitory effect of tacrine on the uptake of organic cations was examined to study the potential transport mechanism of tacrine in the BBB. As expected, the uptake of choline, MPP, TEA, and carnitine by MBEC4 cells was inhibited (e.g., at 100 μM tacrine addition, 51, 37, 58, and 48% reduction for choline, MPP, TEA, and carnitine uptake, respectively; Fig. 3), indicating that these organic cations and tacrine may share a common transport mechanism. Therefore, these observations suggest that tacrine is transported by organic cation transport system(s).

**Identification of mOCT2 and mOCTN2 mRNAs in MBEC4 Cells.** In the literature, the species of organic cation transport systems that are expressed in brain microvessel endothelial cells is (are) somewhat controversial. For example, Slitt et al. (2002) reported that rOCT1 mRNA is expressed in rat brain. In contrast, Gründemann et al. (1997) reported that rOCT1 mRNA is limited to non-neuronal tissue, whereas OCT2 was found in the cerebral region such as the striatum and substantia nigra. In the case of OCT3, whereas the transport system is reported to be present in the brain (Wu et al., 1998), affinity of the transporter for TEA is apparently low (reported K_m value of greater than 2 mM; Dresser et al., 2001). In the study with MBEC4 cells, TEA uptake was readily inhibited by the addition of tacrine (e.g., 58% inhibition in TEA uptake by the addition of 100 μM tacrine), suggesting that OCT3 is not primarily involved in the transport process of tacrine. In addition to these OCT transporters, OCTN2 was reported to be present in brain microvessel endothelial cells (Kido et al., 2001; Friedrich et al., 2003) and transport carnitine (Iinami et al., 2003). For OCTN1, a proton-organic cation exchanger, the transporter is not likely to be functionally efficient, considering the comparable pH values in the systemic circulation and the cytosol of brain microvessel endothelial cells. Therefore, we were primarily interested in the identification of OCT1, OCT2, and OCTN2 in MBEC4 cells. Among the three organic cation transport systems, the mOCT1 message could not be amplified for mouse brain endothelial cells (Fig. 4A), whereas messages for the other two organic cation transport systems were readily amplified (Fig. 4, A and B). In a control study, a RT-PCR procedure for mOCT1 readily amplified in the mouse kidney.

![Fig. 2. Temporal profiles for tacrine concentrations in the plasma (panel A) and striatal ECF (panel B) after an i.v. bolus administration in freely moving rats. Open triangles, 1 mg/kg; open circles, 2 mg/kg; open squares, 5 mg/kg. Data are expressed as the mean ± S.D. of quadruplicate experiments.](https://dmd.aspetjournals.org/347/1/sunget444.png)

**TABLE 2**

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameters</th>
<th>Dose</th>
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<tbody>
<tr>
<td></td>
<td>1 mg/kg</td>
</tr>
<tr>
<td>AUCplasma (mg × min/ml)</td>
<td>8.71 ± 1.50</td>
</tr>
<tr>
<td>V_m (l/kg)</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>AUCbrain (mg × min/ml)</td>
<td>12.5 ± 3.28</td>
</tr>
<tr>
<td>K_D,brain, AUCbrain divided by AUCplasma</td>
<td>14.7 ± 1.5</td>
</tr>
<tr>
<td>Vss (l/kg)</td>
<td>1.75 ± 0.59</td>
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</table>
suggesting that the amplification procedure is adequate for the transporter (data not shown). In addition, amplification of mRNA of rOCT2 and rOCTN2 was successfully carried out in rat brain cDNA (Fig. 4C). Taken together, these observations indicate that OCT2 and OCTN2 are present in the mouse brain endothelial cells and the rat brain.

**Transport of Tacrine in LLC-PK1 Cells in the Presence of Organic Cations.** In a preliminary study, the amount of tacrine transported to the receiving compartment was approximately 10% of the donor compartment throughout the study. In addition, the temporal profile of substrate accumulation in the receiving compartment was proportional to the time of incubation. Collectively, these observations indicate that a sink condition was reasonably maintained in the LLC-PK1 cell study. Since it was indicated that the expression of OCT2 in LLC-PK1 cells is on the basal side (Sweet et al., 2000), tacrine transport from the basal side to the apical side of the cell monolayer was examined. The transport of tacrine (1 μM) was inhibited by the addition of choline (500 μM, 23% inhibition after 30-min transport), MPP (500 μM, 54% inhibition after 30-min transport), and TEA (500 μM, 43% inhibition after 30-min transport) (Fig. 5), suggesting that the tacrine transport system shares a common transport mechanism with choline, MPP, and TEA. This observation is consistent with the inhibition of the uptake of these organic cations in the presence of tacrine in MBEC4 cells (Fig. 3).

Since the subcellular localization of OCTN2 was reported to be the brush border membrane of placental epithelial cells (Lahjouji et al., 2004), apical to basal transport of tacrine (1 μM) was examined for potential involvement of OCTN2 in the transport in LLC-PK1 cells. The addition of carnitine at the concentration of 500 μM led to a diminishment in the apical to basal transport (20% reduction; Fig. 5) of tacrine, consistent with the inhibition of the uptake of carnitine in the presence of tacrine in MBEC4 cells (Fig. 3). Therefore, these observations indicate that the transport of carnitine...
transport mechanism that mediates into and out of barriers between the systemic circulation and tissue (e.g., the brain) has not been studied in the literature. Since tacrine is an organic cation (Fig. 1A), it appeared reasonable to assume that organic cation transporter(s) is (are) involved in the transport. Indeed, the uptake of organic cations was inhibited by the addition of tacrine in MBEC4 cells, an in vitro model of the BBB, and the vectorial transport of tacrine in LLC-PK1 cells was inhibited by the presence of typical substrates for organic cation transport systems (Fig. 5). In addition, our preliminary results indicated that the transport for these organic cations was cross-inhibited in the LLC-PK1 cells by the addition of tacrine (data not shown). Collectively, these observations suggest the involvement of organic cation transport system(s) in the tacrine transport across the rat BBB. Furthermore, since the organic cations used in this study represent model substrates for OCTs and OCTNs, multiple transport mechanisms may exist for the transport of tacrine in the BBB.

Literature information indicates that at least five distinct transport systems for organic cations (i.e., OCT1, OCT2, OCT3, OCTN1, and OCTN2) are operational in mammalian cells (Koepsell, 1998; Dresser et al., 2001). Among these, certain transporters are neither likely to be expressed in the BBB nor likely to be functionally crucial in the BBB. In contrast, mRNAs for mOCT2 and mOCTN2 were readily amplified in samples from MBEC4 cells. Furthermore, rOCT2 and rOCTN2 were detected in rat brain samples, consistent with the identification study in MBEC4 cells. In uptake studies involving rOCT2- or rOCTN2-transfected cells, the accumulation of representative substrates was found to be reduced by the presence of tacrine. Therefore, our findings suggest that the molecular evidence is consistent with the in vivo microdialysis study and the in vitro cell culture study. To our knowledge, this is the first indication that carrier-mediated transport involving multiple organic cation transport systems may mediate the penetration of tacrine across the BBB.

Sweet et al. (2000) reported that the expression of OCT2 was on the basal side of the kidney epithelium. In contrast, uptake activity of choline, MPP, and TEA was detected in MBEC4- and rOCT2-transfected SK-HEP1 cells grown on a plastic support, in which the apical side of the cell systems is primarily exposed to the culture media. Although the underlying mechanism for the apparent discrepancy was not directly investigated in this study, differences between kidney epithelium and brain endothelium/liver epithelium may have contributed to the discrepancy. In addition, considerable discordant results were reported in the literature for the subcellular localization of OCT2 (Grundemann et al., 1997). In the case of rOCT2-transfected SK-HEP1 cells, an enhancement in the accumulation of MPP, a standard OCT2 substrate, was readily apparent, suggesting that the transport system mediated the influx of the organic cation into the cell from the apical side of the cell system.

In this study, the uptake of carnitine was inhibited by the presence of tacrine in rOCTN2-transfected HEK 293 cells at a relatively high concentrations (i.e., greater than 100 μM). The fact that such a high concentration of tacrine is required for the inhibition of carnitine uptake appears to suggest that the affinity of tacrine to the transport system is probably low. Consistent with this observation, the apical to basal transport of tacrine was inhibited by approximately 20% of the control value, with 500 μM carnitine being present in LLC-PK1 cells. Despite the potentially low affinity of the transport for tacrine, the functional and molecular evidence (e.g., inhibition of transports of tacrine by carnitine in LLC-PK1 cells, inhibition of carnitine uptake by tacrine in rOCTN2-transfected cells, and the presence of mOCTN2 and rOCTN2 in MBEC4 cells and rat brain, respectively) are consistent with a partial involvement of OCTN2 in the BBB transport of carnitine and tacrine shares a common mechanism in LLC-PK1 and MBEC4 cells.

**Uptake of Organic Cations in rOCT2-Transfected SK-HEP1 Cells.** The cDNA of rOCT2 was transfected in SK-HEP1 cells in the presence of a cationic liposome. After a 3-week selection period, the expression of rOCT2 was verified by the RT-PCR, and functional expression was accompanied with an approximately 5-fold increase in the uptake of N-[1-14C]MPP transport (data not shown). In rOCT2-transfected cells, the uptake of choline, MPP, or TEA was reduced by the addition of tacrine in a dose-dependent manner (Fig. 6A to C). When eq. 8 was fitted to the uptake results by means of a nonlinear regression analysis, the estimated IC50 values were 0.54 μM, 0.3 μM, and 0.37 μM for choline, MPP, and TEA, respectively, suggesting that the inhibition of MPP uptake by the presence of tacrine was the most pronounced. These observations suggest that rOCT2 mediates the transport of tacrine by sharing a common transport mechanism with choline, MPP, and TEA.

**Uptake of Carnitine in rOCTN2-Transfected HEK 293 Cells.** Functional expression of rOCTN2 was verified by a 4-fold increase in the uptake of carnitine for rOCTN2-transfected HEK 293 cells compared with the control (data not shown). The uptake of carnitine was reduced by the addition of tacrine in rOCTN2-transfected HEK 293 cells in a dose-dependent manner (Fig. 6D). However, only the one highest concentration level (1000 μM final concentration) for tacrine was increased with the dosage, indicating that a carrier-mediated transport is consistent with a partial involvement of OCTN2 in the BBB transport of carnitine and tacrine shares a common mechanism in LLC-PK1 and MBEC4 cells.

**Discussion**

The site of pharmacological action for cholinesterase inhibitors such as tacrine is the brain, and, thus, an understanding of the kinetic mechanism for the penetration of the drug into the brain is likely to have clinical implications. Unfortunately, however, literature information is not clear on the kinetic process(es) for the case of the tacrine penetration. In this study, we found that the partition of the cholinesterase inhibitor from the systemic circulation to the brain ECF decreased with the dosage, indicating that a carrier-mediated transport is involved in tacrine penetration in vivo. Unfortunately, however, the
tacrine. Therefore, the involvement of the transport system in the transport of tacrine across the BBB cannot be ruled out.

In the literature, the microdialysis of tacrine has been applied to study the neuropharmacokinetics with only limited success. For example, the in vivo recovery of tacrine was not evaluated in one study (Telting-Diaz and Lunte, 1993), whereas highly variable in vivo recovery results were obtained in another study (Brundage, 1996). Methods for improving tacrine recovery across a dialysis membrane have not been proposed to date. Without pretreatment of the probe with 9-aminoacridine, the equilibration of tacrine and 9-aminoacridanone across the microdialysis membrane was found to be extremely slow (data not shown). Interestingly, 9-aminoacridine pretreatment greatly improved the rate of equilibration for both tacrine and the retrocalibrator (data not shown). In this study, we did not directly investigate the underlying mechanism involved in the improvement in equilibration for tacrine and the retrocalibrator by 9-aminoacridine pretreatment. However, since 9-aminoacridine is an organic cation and a structural analog of tacrine and retrocalibrator, minimization of the adsorption of tacrine and the retrocalibrator by the addition of 9-aminoacridine may have been contributed to the improvement in recovery across the dialysis membrane.

In this study, the concentration of tacrine in the plasma was found to be in the range of 0.02 to 4.3 $\mu$M. Since a saturable process for tacrine penetration across the BBB was indicated in our in vivo and in vitro studies, the $K_m$ value for the transport is likely to be in the low micromolar range. In a preliminary study, we evaluated in vivo brain uptake clearance for tacrine in rats in the presence and absence of TEA coadministration. The uptake clearance was reduced when TEA was coadministered (data not shown), indicating that the saturable process by organic cation transport systems found in the in vitro experiments is also relevant in vivo.

In summary, the transport of tacrine across the BBB of the rat was found to be saturable in vivo. mRNA for OCT2 was present in mouse brain microvessel endothelial cells and the rat brain. The uptake of typical substrates was inhibited by the addition of tacrine in rOCT2-transfected cells, and the inhibition profile was apparently consistent with that obtained in an in vitro model of the BBB. In addition, the vectorial transport of tacrine was inhibited by the presence of substrates of the transporter in LLC-PK1 cells. Despite the fact that the functional and molecular evidence for OCTN2 was similar to that of OCT2, the affinity of tacrine to OCTN2 was apparently lower than that of OCT2. Therefore, these observations indicate that tacrine transport across the BBB in rats is, in part, mediated by rOCT2. Secondary to the transport, OCTN2 may also be involved in the transport of tacrine in the BBB.

### References


