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Donepezil, Tacrine and α-Phenyl-*n*-*tert*-Butyl Nitrone (PBN) Inhibit Choline Transport by Conditionally Immortalized Rat Brain Capillary Endothelial Cell Lines (TR-BBB)

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In the present study, we have characterized the choline transport system and examined the influence of various amine drugs on the choline transporter using a conditionally immortalized rat brain capillary endothelial cell line (TR-BBB) *in vitro*. The cell-to-medium (C/M) ratio of [^{3}H]choline in TR-BBB cells increased time-dependently. The initial uptake rate of [^{3}H]choline was concentration-dependent with a Michaelis-Menten value, K_m, of 26.2 ± 2.7 μM. The [^{3}H]choline uptake into TR-BBB was Na^+-independent, but was membrane potential-dependent. The [^{3}H]choline uptake was susceptible to inhibition by hemicholinium-3, and tetraethylammonium (TEA), which are organic cation transporter substrates. Also, the uptake of [^{3}H]choline was competitively inhibited with K_i values of 274 μM, 251 μM and 180 μM in the presence of donepezil hydrochloride, tacrine and α-phenyl-*n*-*tert*-butyl nitrone (PBN), respectively. These characteristics of choline transport are consistent with those of the organic cation transporter (OCT). OCT2 mRNA was expressed in TR-BBB cells, while the expression of OCT3 or choline transporter (CHT) was not detected. Accordingly, these results suggest that OCT2 is a candidate for choline transport at the BBB and may influence the BBB permeability of amine drugs.

Key words: Donepezil, tacrine, PBN, Choline transport, Organic cation transporter, Blood-brain barrier, Rat brain capillary endothelial cell line

INTRODUCTION

The blood-brain barrier (BBB), which is formed by the tight junctions of the brain capillary endothelial cells, segregates the circulating blood from brain interstitial fluid, and restricts the penetration of hydrophilic, charged or high molecular weight compounds from blood to brain (Pardridge, 2003; Tamai and Tsuji, 2000). The BBB incorporates various transporters that serve to regulate the entry of endogenous compounds or drugs into the brain (Pardridge, 1998), as well as the efflux of xenobiotics and endogenous compounds such as homovanillic acid (Mori *et al.*, 2003).

Choline is an essential nutrient that plays an important role as a component of membrane phospholipids and a precursor for the synthesis of the neurotransmitter acetylcholine (Klein *et al.*, 1993; Cornford *et al.*, 1978). Since very little choline is synthesized in the brain, choline transport from plasma to brain is required for cellular membrane construction and acetylcholine production (Diamond, 1970). There is *in vivo* (Cornford *et al.*, 1978; Allen and Smith, 2001) and *in vitro* (Sawada *et al.*, 1999; Friedrich *et al.*, 2001) evidence of choline uptake into the brain. These reports suggested that choline is transported from blood to brain across the BBB *via* a carrier-mediated mechanism that is dependent on membrane potential, and the characteristics of choline uptake across the BBB are similar to those of organic cation transporters (OCTs).

The BBB choline transporter (CHT) may be utilized to deliver positively charged quaternary ammonium compounds to the brain across the BBB (Metting *et al.*, 1998). The relationship between transport of basic drugs and choline has been examined (Kang *et al.*, 1990), and it was recently reported that nicotine analogues are transported into the brain *via* CHT (Allen *et al.*, 2003).

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Accordingly, the purpose of the present study was to clarify the expression of the choline transporter (CHT) and/or organic cation transporters (OCTs) at the blood-brain barrier and the interaction of several central nervous system (CNS)-acting drugs with choline transport at the blood-brain barrier. A conditionally immortalized rat brain capillary endothelial cell line, TR-BBB, was used as an *in vitro* model of the BBB.

MATERIALS AND METHODS

Materials

[Methyl-^{3}H]Choline ([^{3}H]choline, 86.0 Ci/mmol) and [carboxyl-^{14}C]inulin ([^{14}C]inulin, 1.92 mCi/g) were purchased from NEN Life Sciences (Boston, MA). Choline, betaine, tetraethylammonium chloride (TEA), L-carnitine, *O*-acetyl-L-carnitine hydrochloride, and hemicholinium-3 were purchased from Sigma Chemical Co. (St Louis, MO). α-Phenyl-*n*-*tert*-butyl nitrone (PBN) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Tacrine, 9-amino-1,2,3,4-tetrahydroacridine hydrochloride, was kindly supplied by Jeil Co. (Seoul, Korea). Donepezil hydrochloride, (±)-2-[(1-benzylpiperidin-4-yl)methyl]-5,6-dimethoxyindan-1-one monohydrochloride, was kindly provided by Daewoong Co. (Seoul, Korea). All other chemicals were commercial products of reagent grade.

Cell culture

TR-BBB cells (passage number 19-23) were grown routinely in type-I collagen-coated tissue culture dishes (Iwaki, Chiba, Japan) at 33°C under 5% CO_2 and 95% air as described previously [13]. For experiments, they were cultured in Dulbecco's modified Eagle's medium (GIBCO, NY) supplemented with penicillin-streptomycin (GIBCO), 10% fetal bovine serum (GIBCO) and 15 μg/L endothelial cell growth factor (Roche, Mannheim, Germany).

Uptake measurements

The [^{3}H]choline uptake study was performed according to the previous report (Hosoya *et al.*, 2000). TR-BBB cells (1×10^5 cells/well) were cultured at 33°C for 2 days on rat tail type-I collagen-coated 24-well plates (Iwaki) and washed with 1 mL of extracellular fluid (ECF) buffer consisting of 122 mM NaCl, 25 mM $NaHCO_3$, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, 10 mM D-glucose and 10 mM Hepes (pH 7.4) at 37°C. Uptake was initiated by applying 200 μL of ECF buffer containing 0.5 μCi of [^{3}H]choline and 0.1 μCi of [^{14}C]inulin to permit correction for bound water at 37°C in the presence or absence of inhibitors. When the influence of Na^+ on the uptake process was to be studied, NaCl and $NaHCO_3$ were replaced with equimolar concentrations of KCl and $KHCO_3$ in the presence of 10 μM valinomycin. After appropriate time periods, the applied solution was removed to terminate uptake and the cells were immersed in ice-cold ECF buffer. The cells were then solubilized in 750 μL of 1 N NaOH. An aliquot (50 μL) was taken for protein assay using a DC protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. The remaining solution (500 μL) was mixed with 5 mL of scintillation cocktail (Hionic-fluor; Packard, Meriden, CT) for measurement of radioactivity in a liquid scintillation counter (LS6500; Beckman, Fullerton, CA)

Data analysis

For kinetic studies, the Michaelis-Menten constant (K_m) and the maximum uptake rate (V_{max}) of [^{3}H]choline were estimated from equation (1):

$$V = V_{max} \cdot C / (K_m + C) \quad (1)$$

where V and C are the initial uptake rate of [^{3}H]choline at 5 min and the concentration of choline, respectively. To analyze the effects of PBN, donepezil and tacrine on the [^{3}H]choline uptake, the inhibitory constant (K_i) was calculated from equation (2):

$$V = V_{max} \cdot C / [K_m \cdot (1 + I/K_i) + C] \quad (2)$$

where I corresponds to the concentration of PBN, donepezil or tacrine, respectively. The K_m, V_{max}, K_i were detemined by employing the non-linear least-squares regression analysis program MULTI (Yamaoka *et al.*, 1981).

Unless otherwise indicated, all data are given as mean ± S.E.M. An unpaired, two-tailed Student's *t*-test was used to determine the significance of differences between group means. Statistical significance of differences among means of several groups was determined by one-way analysis of variance followed by modified Fisher's least-squares difference method and $p<0.05$ was considered statistically significant.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Expression of choline transporter 1 (CHT1), rat organic cation transporter 1 (rOCT1), rat organic cation transporter 2 (rOCT2) and rat organic cation transporter 3 (rOCT3) by TR-BBB cells was analyzed by RT-PCR. The sequences of sense and antisense primers and the experimental conditions are given in Table I. Total mRNA was isolated by the acid-phenol procedure using ISOGEN (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) according to the manufacturer's protocol. The first standard cDNA reaction was performed using ReverTra Ace M-MLV reverse transcriptase (ReverTra Ace, Toyobo Co., Ltd., Osaka, Japan). RT-PCR was performed with TAKARA Ex Taq™ (Takara Shuzo Co., Ltd., Shiga, Japan) according to the manufac-

Table I. Primers used in reverse transcription-polymerase chain reactions to analyze the expression of rat choline transporter 1 (rCHT1) and rat organic cation transporters (rOCTs)

Gene		Sequence (5' to 3')	Accession	Condition no. (°C)
rCHT1	Sense primers	CTGAGCTCTGACCTTGTCTA	NM_053521	57
	Antisense primers	CCTCTGGACTGGAATCAACA		
rOCT1	Sense primers	CATCTGTGTCCGGTGTGCTA	U76379	57
	Antisense primers	ATGACGGTGTGCTTCCTCAG		
rOCT2	Sense primers	GGAGTGGCCTATGTGATTCC	D83044	57
	Antisense	CGGTCGATGGTGAGGATGAT		
rOCT3	primers Sense primers	TATGCAGCGGACAGATACGG	AF055286	57
	Antisense primers	CCTTGATACACCACGGCACT		

turer's instructions.

PCR was carried out using the following protocol. After an initial melting temperature of 85°C for 5 min, primers were added according to the hot start method. Amplification cycles of 1 min of denaturation at 94°C, 1 min of annealing at 57°C, and 1 min of extension at 72°C were repeated, followed by a final extension. The products were subjected to electrophoresis on 5% acrylamide gel, then the gel was stained with ethidium bromide, and bands were visualized under ultraviolet light.

RESULTS

Characterization of choline transport in TR-BBB cells

To characterize choline transport at the BBB, [^{3}H]choline uptake was evaluated using TR-BBB cells as an *in vitro* BBB model (Terasaki *et al.*, 2003). As shown in Fig. 1, [^{3}H]choline uptake by TR-BBB cells exhibited a time-dependent increase and was linear for at least 60 min. When TR-BBB cells were depolarized by replacement of Na^+ with K^+ in the ECF buffer and addition of 10 μM valinomycin, as an ionophore of K^+, the uptake of [^{3}H]choline was significantly decreased. This result indicated that [^{3}H]choline uptake is membrane potential-dependent.

The saturation kinetics of choline uptake was analyzed over the concentration range of 0.031-500 μM. [^{3}H]Choline uptake by TR-BBB took place in a concentration-dependent manner (Fig. 2A). The Eadie-Scatchard plot (Fig. 2B) gave a single straight line, indicating a single saturable process. Kinetic analysis provided the Michaelis-Menten constant (K_m) of 26.2 ± 2.7 μM and the maximal velocity (V_{max}) of 397 ± 14 pmol/(mg protein · min). These results indicate that choline transport at the BBB is a saturable carrier-mediated process. The dose-response relationship for the inhibition of unlabeled choline, hemicholinium-3, and PBN on [^{3}H]choline uptake was examined in TR-BBB cells and the results are shown in Fig. 3. The unlabeled choline inhibited the uptake of [^{3}H]choline with an IC_{50} value (i.e., concentration at which the inhibition was 50%) of 9.40 μM. The choline analogue hemicholinium-3 inhibited the uptake with an IC_{50} value of 37.4 μM. The IC_{50} value for PBN was 1.20 mM.

The effect of substrates and inhibitors of the choline transporter on [^{3}H]choline uptake by TR-BBB cells is summarized in Table II. [^{3}H]Choline uptake was inhibited by more than 80% by choline and hemicholinum-3 (substrates for the choline transporter) and by up to 35% by tetraethylamonium (TEA) as a substrate for the organic cation transporter. Betaine and L-carnitine had no effect on [^{3}H]choline uptake.

Effects of various drugs on [^{3}H]choline uptake by TR-BBB cells

The effects of several drugs on [^{3}H]choline uptake by TR-BBB cells are summarized in Table III. [^{3}H]Choline uptake was inhibited by more than 90% by verapamil, an OCT substrate. Donepezil hydrochloride and tacrine, used to treat Alzheimer's disease, also inhibited [^{3}H]choline uptake by more than 90%. The positively charged antioxidant PBN also inhibited [^{3}H]choline uptake by about 75%. [^{3}H]Choline uptake was inhibited by 40% by acetyl L-carnitine. However, several acidic drugs such as probenecid and benzylpenicilin had no effect on [^{3}H]choline uptake.

The Lineweaver-Burk plot showed that the two lines of choline uptake in the presence or absence of 1 mM PBN intersected the ordinate axis (Fig. 4A). This indicated that PBN competitively inhibited choline uptake with a K_i of 180 μM. Also, Fig. 4B and Fig. 4C show that the two lines of the choline uptake in the presence or absence of 0.1 mM donepezil or 0.2 mM tacrine intersected the ordinate axis. These results indicated that donepezil and tacrine also competitively inhibited choline uptake with K_i values of 274 μM and 251 μM, respectively.

Analysis of the expression of organic cation transporters (OCTs) in TR-BBB cells

The expression of rCHT1 and rOCTs (1-3) mRNAs in TR-BBB cells was analyzed by RT-PCR using total mRNA isolated from TR-BBB and rat brain and primers

that are specific for rCHT1, rOCT1, rOCT2 and rOCT3 (Fig. 5). Although products derived from rOCT1 at 489 bp, rOCT2 at 439 bp and rOCT3 at 580 bp were amplified from rat brain only that from rOCT2 was amplified in TR-BBB cells. TR-BBB cells also do not express the Na^+-dependent choline transporter rCHT1 (492 bp product). The nucleotide sequence of the bands of rat brain and TR-BBB cells showed 100% homology with the corresponding regions of rat CHT1, OCT1, OCT2 and OCT3 (data not shown).

DISCUSSION

Our results show that carrier-mediated choline transport is significantly inhibited by donepezil, tacrine and α-phenyl-*n*-*tert*-butyl nitrone (PBN) in TR-BBB cells, which are considered to be a model of the blood-brain barrier, and rOCT2 contributes, at least in part, to choline transport in these cells. The mRNA expression of the choline transporter (CHT) was not detected in TR-BBB cells.

TR-BBB was established from transgenic rats harboring temperature-sensitive SV40 large T antigen gene, and grew well at 33°C (Hosoya *et al.*, 2000). TR-BBB cells express several transporter genes including mdr1a, large neutral amino acid (LAT1) and GLUT-1 (Hosoya *et al.*, 2000). Moreover, mRNAs of tight-junction strand proteins such as claudine-5, occludin, and junctional adhesion molecule are also expressed in TR-BBB cells (Hosoya *et al.*, 2000). TR-BBB cells are thought to be one of the best *in vitro* BBB models in terms of reflecting *in vivo* transporter gene expression and transport activities (Pardridge, 2004; Terasaki *et al.*, 2003). In the present study, the [^{3}H]choline uptake was increased time-dependently and was independent of Na^+ (Table II). On the other hand, the uptake of [^{3}H]choline was significantly decreased by the replacement of Na^+ with K^+ in the ECF buffer and addition of 10 mM valinomycin (Fig. 1). Sawada et al. reported that when cultured mouse brain capillary endothelial cells (MBEC4) were depolarized by replacement of Na^+ with K^+ in the incubation buffer and addition of 10 μM valinomycin, the uptake of choline was significantly decreased. In TR-BBB cells, very similar results were obtained. This finding suggests that transport of choline in TR-BBB is membrane potential-dependent. The [^{3}H]choline uptake was inhibited by hemicholinium-3, which is a competitive inhibitor of the choline transporter (Table II). The K_m and V_{max} values for [^{3}H]choline uptake by TR-BBB cells were about 26 μM and 397 pmol/(mg protein · min), respectively (Fig. 2). These results indicate that TR-BBB cells possess a high-affinity choline uptake system, which is dependent upon membrane potential and sensitive to the choline analog hemicholinium-3.

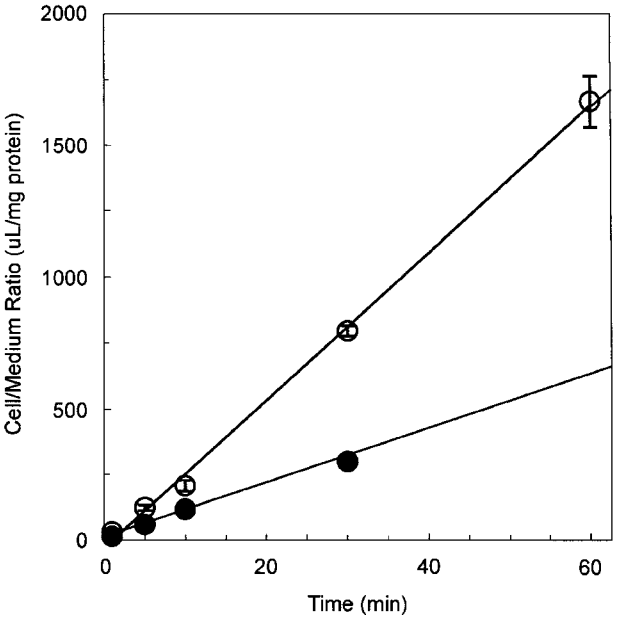

Fig. 1. Time-course of [^{3}H]choline uptake by TR-BBB cells. [^{3}H]Choline (11.6 nM) uptake was performed at 37°C in ECF buffer (○) or buffer in which Na^+ was replaced by K^+ and which contained 10 μM valinomycin (●). Each point represents the mean ± S.E.M (n=4).

Table II. Effect of several compounds on [^{3}H]choline uptake by TR-BBB cells

Inhibitor	Concentration (mM)	Uptake of [^{3}H]Choline (% of control)
Control	0	100 ± 10
Na^+-free conditions	0	98.7 ± 2
Choline	10	4.15 ± 0.67**
Hemicholinium-3	0.1	19.4 ± 2.2**
Betaine	2	82.3 ± 10.4
TEA	2	64.9 ± 6.6*
L-Carnitine	2	88.1 ± 7.9

[^{3}H]Choline (11.6 nM) uptake by TR-BBB cells was measured in the absence (control) or presence of an inhibitor at 5 minutes and 37°C. Each value represents the mean ± S.E.M. (n = 3-4). *p<0.05, **p< 0.001; significantly different from control.

Choline transport at the BBB has been demonstrated *in vivo* and *in vitro* to be saturable and carrier-mediated. Early *in vivo* choline uptake studies gave a K_m value of 310 μM (Conford *et al.*, 1978) or 180 μM (Kang *et al.*, 1990), but Allen and Smith reported that the choline uptake was sodium ion-independent and sensitive to hemicholinium-3, with a K_m value of 42 μM, which is in the range expected for high-affinity mechanisms. In addition, choline uptake studies into the brain were performed in *in vitro* models. Both MBEC4 cells and the rat brain capillary endothelial cell lines (RBE4) were found to show Na^+-independent, saturable choline uptake with a K_m value of about 20 μM, in good agreement with the results of rat

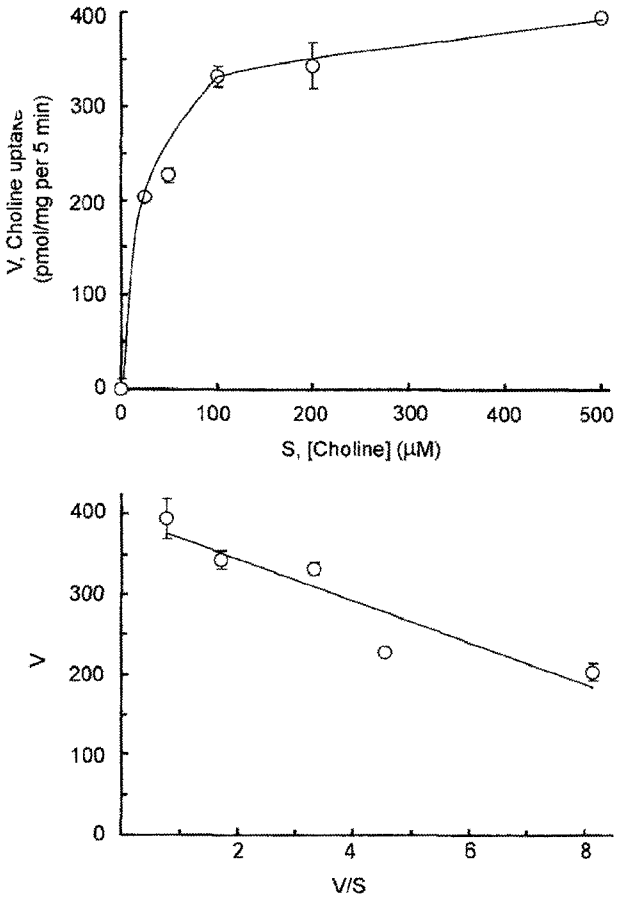

Fig. 2. Saturation kinetics of choline uptake. Uptake of choline was measured in confluent TR-BBB cells with 5 minutes incubation in the presence of NaCl at pH 7.4, 37°C. The concentration of choline was varied from 0-500 μM by changing the concentration of unlabeled choline with the constant concentration of labeled choline of 11.6 nM (A). The data are shown as an Eadie-Hofstee plot. The values of v and s represent initial pseudolinear uptake (pmol/(mg protein · min) and choline concentration (μM), respectively (B). The K_m is 26.2 μM and the V_{max} is 397 pmol/mg of protein per 5 minutes. The data represents the mean ± S.E.M (n=4).

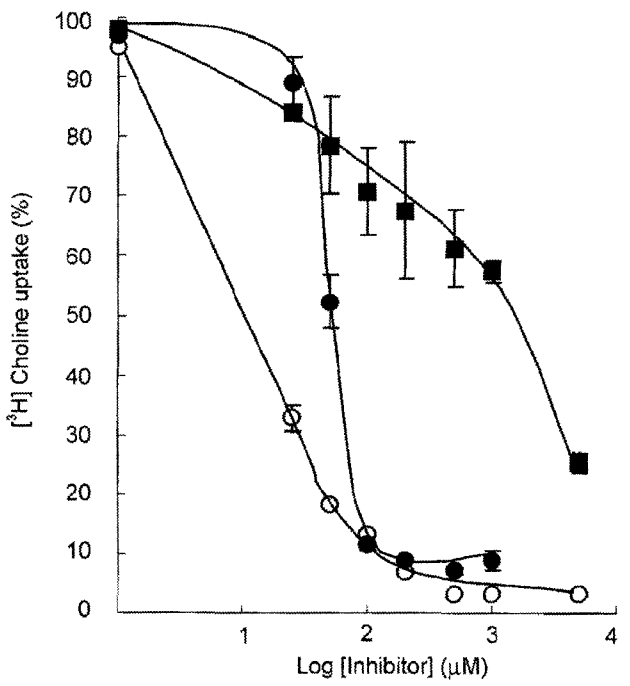

Fig. 3. Dose-response relationship for the inhibition of [^{3}H]choline uptake by several organic cations. Uptake of [^{3}H]choline (11.6 nM) was measured in confluent TR-BBB cells with a 5 minutes incubation in the presence of NaCl at pH 7.4 either in the absence (control) or presence of increasing concentrations of α-phenyl-n-tert-butyl nitrone (PBN), hemicholinium-3. Results (mean ± S.E.M) are given as percent of control uptake measured in the absence of inhibitors. ○: unlabeled choline, ●: hemicholinium-3, ■: PBN. The IC_{50} values for choline, hemicholinium-3 and PBN are 9.40 μM, 37.2 μM and 1.20 mM.

brain *in situ* perfusion studies (Sawada *et al.*, 1999; Friedrich *et al.*, 2001). The characteristics of the choline transport system in TR-BBB cells were very similar to these reported recently. The K_m value is in good agreement with the apparent K_m of about 20 μM in both MBEC4 cells and RBE4 cells. Also, hemicholinum-3 inhibited the uptake of choline in RBE4 cells with an IC_{50} value of about 50 μM, which is similar to the IC_{50} value (about 40 μM) found in TR-BBB cells (Fig. 3). These results suggest that the choline transport in TR-BBB cells is the same high-affinity transport system as in MBEC4 cells and RBE4 cells.

Previous reports have presented evidence that various amine compounds are transported into the brain *via* a BBB choline transport system. The basic amine drug, eperisone, competitively inhibited choline uptake in rats with a K_i of 455 μM (Kang *et al.*, 1990). Derivatives of lobeline and isoarecolone bind to the BBB choline transport system and may enter the brain via this transport system (Metting *et al.*, 1998). Donepezil hydrochloride and tacrine are reversible cholinesterase inhibitors that exhibit high specificity for centrally active cholinesterase (Rho and Lipson, 1997; Hartvig *et al.*, 1990; Telting-Diaz and Lunte, 1993). PBN, a nitrone-based spin trapping agent has been proposed as a therapeutic agent for stroke (Knecht and Mason, 1993; Zhao *et al.*, 1994). The brain distribution of these drugs is relatively high, but the transport mechanism(s) remains unclear (MaNally *et al.*, 1996; Matsui *et al.*, 1999). Our results show that PBN, a cationic nitrone derivative, donepezil hydrochloride, a benzylpiperidine derivative, and tacrine, an acridine-based drug, competitively inhibited choline uptake with K_i values of 274 μM, 251 μM and 180 μM, respectively (Fig. 4). Thus, basic amine drugs such as donepezil hydrochloride, tacrine and PBN, ma be delivered into the brain *via* the choline transpc system at the BBB, though this requires confirmation. T maximal plasma concentrations of donepezil hydrochlor and tacrine at the steady states were 61 ng/mL (Tise

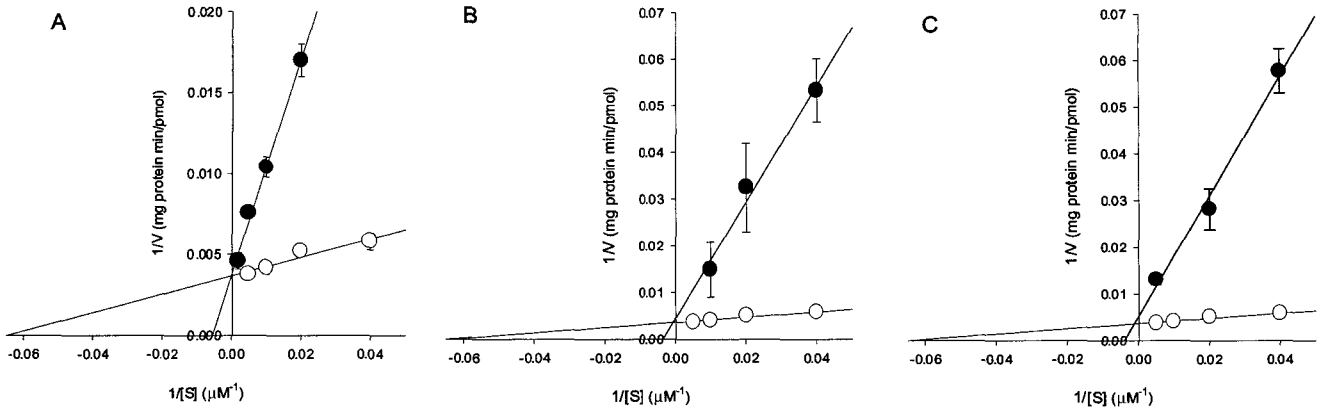

Fig. 4. Lineweaver-Burk plots of choline uptake by TR-BBB cells showing competitive inhibition by several drugs. [^{3}H]Choline (11.6 nM) uptake was performed in the presence (●) of 1 mM PBN (A), 0.1 mM donepezil (B) and 0.2 mM tacrine (C) or the absence (○) at 37°C for 5 min. Each point represents the mean ± S.E.M. (n = 3-4). The K_m for choline uptake is 15.5 μM. The K_i values are 180 μM for PBN, 274 μM for donepezil and 251 μM for tacrine.

al., 1998) and 30 ng/mL (Johansson *et al.*, 1996), respectively, and plasma protein binding rates of donepezil hydrochloride and tacrine were 95% and 55%, respectively (Parfitt *et al.*, 1999). Therefore, donepezil hydrochloride and tacrine may not significantly inhibit choline transport via the choline transport system at the BBB. *In vivo* studies using the brain uptake index (BUI) method (Pardridge and Oldendorf, 1977; Cornford *et al.*, 1978), have suggested that a BBB choline transport system is present at the brain capillary luminal membrane, but in vitro studies using isolated cerebral microvessels (Galea and Estrada, 1992; Kang *et al.*, 1990) and cultured brain endothelial cells (Sawada *et al.*, 1999; Gomez *et al.*, 1993), indicated that the transporter may be localized only at the capillary abluminal membrane. Our *in vivo* BEI results for choline (manuscript in preparation) indicate that the inhibitory effect of these drugs may be seen at both sides of the brain capillary membrane. However, our unpublished data show that choline is eliminated from the brain to the blood across the BBB *via* a carrier-mediated efflux transport system. Tacrine and PBN significantly inhibited [^{3}H]choline efflux by about 40% and 50%, respectively. Therefore these drugs may play a role in maintaining choline concentration in the brain.

Three major organic cation transporters (OCTs), OCT1, OCT2 and OCT3, have been isolated and their transport functions characterized. In the rat, OCT1 was found in the kidney, liver and intestine (Grundemann *et al.*, 1994; Koepsell *et al.*, 1999) OCT2 was located in the kidney and brain (Saito *et al.*, 1996; Grundemann *et al.*, 1998) and OCT3 was found in the kidney, intestine, heart, placenta and brain (Kekuda *et al.*, 1998). OCT1 and OCT2 are independent of Na^+ and pH, and dependent on membrane potential (Grundemann *et al.*, 1994; Saito *et al.*, 1996). Also, choline is not substrate of OCT3, but is a substrate of OCT1 and OCT2 (Kekuda *et al.*, 1998). A Na^+ and Cl^--dependent, hemicholinium-3-sensitive, high-affinity choline transporter, designated CHT1, has recently been cloned (Okuda *et al.*, 2000). CHT1 is thought to be unique to cholinergic neurons in several regions of the brain. Our data showed that the choline transport characteristics resemble those of the OCT family, so we investigated the expression of several OCTs and CHT1 in TR-BBB cells by RT-PCR analysis. We found that OCT1-3 and CHT1 were expressed in the rat brain tissue (Fig. 5), but other reports on OCT expression in brain are conflicting (Grundemann *et al.*, 1997; Gorboulev *et al.*, 1997; Wu *et al.*, 1998). TR-BBB cells expressed OCT2, but not OCT1, OCT3 and CHT1 (Fig. 5). The lack of expression of rCHT1 in TR-BBB cells is consistent with the present findings that there is no detectable Na^+-dependent choline uptake in these cells. Although these RT-PCR results for TR-BBB cells are different from those for RBE4 cells, which express low levels of rOCT1, neither of the cell lines expresses rCHT1 mRNA (Friedrich *et al.*, 2001). According to Sweet *et al.*, rOCT2 was also detected by RT-PCR in the choroid plexus and mediated choline transport across the ventricular

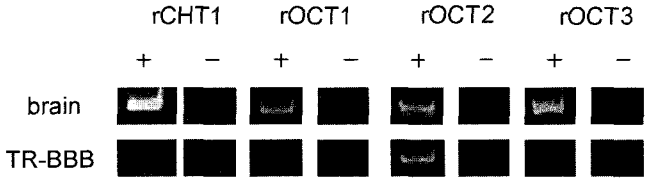

Fig. 5. Detection of rCHT1 and rOCTs by RT-PCR in TR-BBB cells and brain tissues. Total mRNA (1 μg) was reverse-transcribed and cDNA (0.1 μg) was amplified by PCR. Products were electrophoresed on 5% acrylamide gel and visualized by ethidium bromide staining. Products of isolated cells were observed at the expected sizes. (+) and (-) represent the presence or absence of reverse transcriptase, respectively.

Table III. Effect of several drugs on [^{3}H]choline uptake by TR-BBB cells

Inhibitor	Concentration (mM)	Uptake of [^{3}H]Choline (% of control)
Control	0	100 ± 10
Verapamil	2	2.49 ± 0.53**
L-Acetyl carnitine	2	60.6 ± 2.8*
α-Phenyl-n-tert-butyl nitrone (PBN)	5	23.8 ± 1.7**
Donepezil	0.5	2.63 ± 0.38**
Tacrine	2	0.62 ± 0.06**
Probenecid	2	74.6 ± 4.8
Benzyl penicillin	2	105 ± 6

[^{3}H]Choline (11.6 nM) uptake by TR-BBB cells was measured in the absence (control) or presence of an inhibitor at 5 minutes and 37°C. Each value represents the mean ± S.E.M (n = 3-4). *p<0.05, **p< 0.001 significantly different from control.

membrane of the choroid plexus. Taken together, the results suggest that OCT2 may be involved in uptake from plasma to brain of choline and cationic drugs such as donepezil, tacrine and PBN. Recently, it was reported that another organic cation transporter, OCTN2, has a transport function for organic cations as well as carnitine, but choline had a weak inhibitory effect on carnitine uptake (Wu *et al.*, 1999). In the present study, [^{3}H]choline uptake was not significantly inhibited by L-carnitine, but was slightly inhibited b acetyl L-carnitine (Table III). These data suggest that acetyl L-carnitine is delivered into the brain *via* the choline transport system at the BBB, and further studies are warranted to confirm this.

In conclusion, our results suggest that choline is transported across the BBB *via* OCT2, and this transporter may also be involved in the transport of basic drugs such as donepezil hydrochloride, tacrine and PBN into the brain across the BBB. These findings show that compounds structurally related to choline may exhibit increased brain distribution owing to uptake *via* the choline transport system.

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