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Carnosine uptake in rat choroid plexus primary cell cultures and choroid plexus whole tissue from PEPT2 null mice

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Abstract

PEPT2 is functionally active and localized to the apical membrane of rat choroid plexus epithelial cells. However, little is known about the transport mechanisms of endogenous neuropeptides in choroid plexus, and the role of PEPT2 in this process. In the present study, we examined the uptake kinetics of carnosine in rat choroid plexus primary cell cultures and choroid plexus whole tissue from wild-type ($PEPT2^{+/+}$) and null ($PEPT2^{-/-}$) mice. Our results indicate that carnosine is preferentially taken up from the apical as opposed to basolateral membrane of cell monolayers, and that basolateral efflux in limited. Transepithelial flux of carnosine was not distinguishable from that of paracellular diffusion. The apical uptake of carnosine was characterized by a high affinity ($K_m = 34$ μM), low capacity ($V_{max} = 73$ pmol/mg protein/min) process, consistent with that of PEPT2. The non-saturable component was small ($K_d = 0.063$ μL/mg protein/min) and, under linear conditions, was only 3% of the total uptake. Studies in transgenic mice clearly demonstrated that PEPT2 was responsible for over 90% of carnosine's uptake in choroid plexus whole tissue. These findings elucidate the unique role of PEPT2 in regulating neuropeptide homeostasis at the blood–cerebrospinal fluid interface.

Keywords: carnosine, choroid plexus, neuropeptides, PEPT2, transport.

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Carnosine (β-alanyl-L-histidine) is present in muscle and the central nervous system. Although the physiological function of carnosine is not fully understood, many putative roles have been assigned to this dipeptide. In this regard, it may act as a neuroprotective agent against free radicals (Boldyrev *et al*. 1997), inhibit protein glycosylation from reactive aldehydes (Hipkiss *et al*. 1998), or act as a scavenger for reactive oxygen species (Stvolinsky *et al*. 1999). Carnosine reduces the toxicity of β-amyloid, the primary neurotoxin in Alzheimer's disease (Preston *et al*. 1998), and prevents β-amyloid aggregation (Hipkiss 1998). It has also been implicated in protection from metal-induced cell toxicity (Horning *et al*. 2000). Finally, carnosine is present in high concentrations (1–2 mM) in the olfactory bulb (Margolis 1974) and may serve as a neurotransmitter in this region (Marchis *et al*. 2000).

PEPT2, a proton-coupled oligopeptide transporter, is responsible for the high-affinity symport of di-/tripeptides across biological membranes via an inwardly directed electrochemical proton gradient. Although initially cloned from human kidney (Liu *et al*. 1995), this peptide transporter was subsequently cloned from rat brain and found to be identical to rat kidney PEPT2 (Wang *et al*. 1998). PEPT2 transcripts are found throughout the brain and abundantly expressed in epithelial cells of the choroid plexus (Berger and Hediger 1999). Moreover, molecular and functional studies conclusively demonstrate that PEPT2 protein is expressed in choroid plexus tissue and, in particular, on the apical membrane surface of choroidal epithelia (Novotny *et al*. 2000; Teuscher *et al*. 2000, 2001; Shu *et al*. 2002). In this capacity, PEPT2 is believed to act as an efflux pump in

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Abbreviations used: aCSF, artificial CSF; BCSFB, blood–CSF barrier; DMEM, Dulbecco's modified Eagle's medium; GABA, γ-amino-n-butyric acid; GlyGln, glycylglutamine; GlySar, glycylsarcosine; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; NMN, N^1-methylnicotinamide; PAH, p-aminohippuric acid; SITS, 4-acetamido-4′-isothiocyanatostilbene-2, 2′-disulfonic acid; TCA, taurocholic acid; TEA, tetraethylammonium.

the removal of neuropeptides and peptide fragments from cerebrospinal fluid (CSF).

Still, a clear understanding of neuropeptide transport at the choroid plexus is lacking. This is due, in part, to the fact that most choroid plexus studies were performed using a synthetic, model dipeptide (i.e. glycylsarcosine), non-physiologic buffer conditions (e.g. Tris-Mes, acidic pH, low sodium), and whole tissue uptakes that could neither differentiate membrane sidedness nor directionality. In addition, the presence of two peptide/histidine transporters in brain (i.e. PHT1 and PHT2) (Yamashita *et al*. 1997; Sakata *et al*. 2001) makes it difficult to accurately determine the significance of PEPT2 relative to other (peptide) transporters. Notwithstanding this uncertainty, it was reported that PEPT2 accounted for about 60% of the total uptake of carnosine in whole tissue rat choroid plexus (Teuscher *et al*. 2001). However, this conclusion is quite tenuous, as suggested by other studies in PEPT2 null mice (Shen *et al*. 2003).

With this in mind, we used rat choroid plexus epithelial cells in primary culture to investigate the peptide-mediated transport mechanisms of an endogenous neuropeptide, carnosine, at the blood–CSF interface. We also evaluated the whole tissue choroid plexus uptake of carnosine in wild-type and PEPT2-deficient mice. Taken as a whole, these results indicate that apical PEPT2 is the predominant transporter in regulating neuropeptide homeostasis in cerebrospinal fluid.

Experimental procedures

Materials

[3H]Carnosine (9 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA, USA) and [^{14}C]mannitol (53 mCi/mmol) was purchased from American Radiolabeled Chemicals (St Louis, MO, USA). Amino acids (β-alanine and L-histidine), carnosine, glycylglutamine (GlyGln), glycylsarcosine (GlySar), 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), taurocholic acid (TCA), γ-amino-n-butyric acid (GABA), p-aminohippuric acid (PAH), N^1-methylnicotinamide (NMN), tetraethylammonium (TEA) and choline were purchased from Sigma (St Louis, MO, USA). Other chemicals were obtained from standard sources and were of the highest quality available.

Culture medium and uptake buffers

The cell culture medium consisted of Dulbecco's modified Eagle's medium (DMEM)/F-12 (1 : 1) supplemented with 10% (v/v) fetal bovine serum, 15 mM HEPES, 2 mM L-glutamine, 100 units/mL penicillin G sodium, 1 μg/mL streptomycin sulfate, 25 μg/mL gentamicin, 5 mg/mL insulin, 10 ng/mL epidermal growth factor, 2 μg/mL hydrocortisone, 5 μg/mL transferrin, and 5 ng/mL sodium selenite (pH 7.3) (all reagents from Life Technologies, Rockville, MD, USA).

Experiments were performed in normal-Na^+ artificial cerebrospinal fluid (aCSF) and low-Na^+ aCSF. The normal-Na^+ aCSF buffer (about 310 mOsm/kg) contained 127 mM NaCl, 20 mM $NaHCO_3$, 2.4 mM KCl, 0.5 mM KH_2PO_4, 1.1 mM $CaCl_2$, 0.85 mM $MgCl_2$, 0.5 mM Na_2SO_4, and 5.0 mM glucose. In the low-Na^+ aCSF buffer, NaCl and $NaHCO_3$ were replaced with choline chloride and choline bicarbonate, respectively, producing a 1.0 mM sodium solution due to the presence of Na_2SO_4. Both buffers were adjusted to pH 7.4 with HCl.

Primary culture of choroid plexus epithelial cells

Primary cultures of rat choroid plexus epithelial cells were prepared from 1 to 2 day-old neonatal rats using an established method (Strazielle and Ghersi-Egea 1999; Shu *et al*. 2002). Cells were isolated from lateral ventricles and seeded on laminin-coated Transwell-Clear filter inserts (12-mm diameter, 0.4-μm pore size; Costar Plastics, Cambridge, MA, USA). The culture medium was changed at 48–72 h after seeding and every other day thereafter. Cells were grown in a sterile incubator at 37°C, 95% relative humidity and 5% CO_2. Cultures were inspected visually for epithelial growth (cobblestone appearance) on a weekly basis. Confluence occurred at 5–7 days post-seeding. Transepithelial electrical resistance measurements were performed prior to experimentation (using a Millicell-ERS; Millipore Corp., Bedford, MA, USA) as a measure of monolayer integrity. Values of 150 $\Omega \bullet cm^2$ were reached at 10–14 days post-seeding and were considered sufficient for experimentation.

Carnosine intracellular accumulation and transepithelial transport in cell cultures

Cells were incubated apically (0.4 mL) and basolaterally (1.2 mL) in uptake buffer (normal-Na^+ aCSF or low-Na^+ aCSF) at 37°C for 10 min prior to experimentation. The buffer was removed and fresh uptake buffer, containing 0.4 μCi [^{3}H]carnosine and 0.1 μCi [^{14}C]mannitol, was added to the apical (0.4 mL) or basolateral (1.2 mL) chamber; control buffer (no radioisotope) was added to the opposite chamber. Potential inhibitors (1 mM) were added to the same side as carnosine. The cells were then incubated at 37°C for the indicated time. To measure transepithelial transport, a 100 μL sample was taken from the opposite chamber and placed in a scintillation vial. To measure intracellular accumulation, the uptake buffer was aspirated at the end of the incubation period. Three successive rapid washings with ice-cold uptake buffer were then performed. The filter and monolayer were detached and placed in a scintillation vial with 0.5 mL of 0.2 M NaOH and 1% sodium dodecyl sulfate (SDS). Scintillation cocktail [EcoLite(+); ICN, Costa Mesa, CA, USA] was added to the solubilized cells and radioactivity was determined in the sample using a dual-channel liquid scintillation counter (Beckman LS 6000; Fullerton, CA, USA). The protein content of the solubilized cell monolayers was determined by the method of Bradford (1976), using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with immunoglobulin G as the standard.

Carnosine uptake data were corrected for filter binding and extracellular content, as described previously (Teuscher *et al*. 2000). The transepithelial transport of carnosine was corrected for paracellular flux, as estimated by mannitol (Shu *et al*. 2002).

Efflux experiments in cell cultures

Cells were washed twice with normal-Na^+ aCSF buffer and then incubated apically (0.4 mL) and basolaterally (1.2 mL) in this buffer

at 37°C for 10 min prior to experimentation. The buffer was removed and fresh uptake buffer containing 1 μM [^{3}H]carnosine was added to the apical chamber (0.4 mL); control buffer (no radioisotope) was added to the basolateral chamber. The cells were then incubated at 37°C for 2 hours. The cells were removed and washed six times with warm buffer. Fresh normal-Na^+ aCSF buffer was then added to the apical (0.4 mL) and basolateral (1.2 mL) chambers. At the indicated times, a 100-μL aliquot was sampled from the apical and basolateral chambers, and added to a scintillation vial. At the final time point, the cell monolayers were washed and removed, and placed in a scintillation vial with lysis buffer. Scintillation cocktail [Ecolite(+); ICN, Costa Mesa, CA, USA] was added to the samples and radioactivity determined using a dual-channel liquid scintillation counter (Beckman LS 6000). Efflux was expressed as the per cent of total substrate in the cells after loading.

Carnosine stability in cell cultures

Choroid plexus epithelial cells were incubated apically with 1 μM [^{3}H]carnosine for 15, 30, 60 and 120 min. At the end of incubation, media from the apical and basolateral compartments were aspirated and saved for analysis. The cell monolayers were washed three times with ice-cold buffer and solubilized in 0.5 mL of 0.2 M NaOH and 1% SDS. The samples were then analyzed by high-performance liquid chromatography (HPLC), and the concentration of radiolabeled carnosine and its potential degradation product, histidine, was determined. The stability of carnosine was evaluated by its recovery and the appearance of histidine following incubation. Results were reported from three separate experiments.

An analytical method was developed based on work done by O'Dowd *et al*. (1990). Carnosine and histidine were detected using an HPLC system consisting of a pump (Waters, Model 616, Milford, MA, USA), a controller (Waters, Model 600S), a reversed-phase column (Hypersil ODS, C-18, 5 μm, 250 mm × 4.6 mm, Alltech, Deerfield, IL, USA) and a radiochromatography detector (FLO-ONE 500TR, Packard Instrument Co., Meriden, CT, USA). The mobile phase was comprised of 0.1 M Na_2HPO_4 and 0.13% heptafluorobutryic acid, and pumped isocratically at 1 mL/min at room temperature (23°C). Retention times for histidine and carnosine were 6.9 and 18.5 min, respectively.

Endogenous carnosine

Skeletal muscle (600 mg), olfactory bulb (450 mg), choroid plexus (70 mg), cerebrospinal fluid (200 μL) and plasma (3 mL) were obtained from anesthetized adult female rats, and processed accordingly (O'Dowd *et al*. 1990*)*. In brief, tissue samples were homogenized in 2 mL of water. An 8-mL aliquot of ethanol was added to the homogenized tissue and body fluid samples, and the mixture was vortexed and then incubated at 60°C for 90 min. After incubation, the precipitate was pelleted by centrifugation (2000 *g*, 15 min). The supernatant was removed, evaporated to dryness, and then reconstituted in mobile phase. Carnosine was detected at 210 nm using a UV spectrophotometer (Spectroflow 783; ABI Analytical, Chestnut Ridge, NY, USA) coupled to HPLC (as described above).

Carnosine uptake in isolated choroid plexuses of $PEPT2^{+/+}$ and $PEPT2^{-/-}$ mice

Whole tissue choroid plexus uptakes were performed in 30–50-day-old $PEPT2^{+/+}$ (wild-type) and $PEPT2^{-/-}$ (null) mice, as described previously for GlySar (Shen *et al*. 2003). In brief, lateral and fourth ventricle choroid plexuses were harvested from anesthetized mice, transferred to pre-weighed eppendorf tubes and re-weighed to 0.1 mg accuracy using an electronic balance (Mettler AE100; Toledo, OH, USA) to determine plexus weight. They were then immediately transferred to normal-Na^+ aCSF buffer (pH 7.4) which was continuously bubbled with 5% CO_2, 95% O_2. After a 5-min recovery period, the choroid plexuses were transferred to 0.95 mL of fresh buffer for 0.5 min. Uptake was initiated by addition of 0.05 mL of normal-Na^+ aCSF buffer containing about 0.2 μCi of [^{3}H]carnosine (plus 20 μM unlabeled carnosine) and 0.2 μCi of [^{14}C]mannitol (an extracellular marker), resulting in a final carnosine concentration of 1.0 μM. Uptake was terminated after 2 min by transferring the plexuses to ice-cold buffer and filtering under reduced pressure. The filters (118-μm mesh, Tetko, Kansas City, MO, USA) were washed three times with the same buffer. The filters and choroid plexuses were then soaked in 0.33 mL of 1 M hyamine hydroxide (a tissue solubilizer) for 30 min before the addition of scintillation cocktail (Cytoscint) and counting with a dual-channel liquid scintillation counter (Beckman LS 3801). Similar uptake studies were performed for carnosine in low-Na^+ aCSF buffer (pH 7.4). The uptake of radiolabeled carnosine into choroid plexus (V_d, in μL/mg of wet tissue weight) was calculated as described previously (Teuscher *et al*. 2000).

All of the rat and mice experiments were carried out in accordance with animal care guidelines and with the approval of the University of Michigan Committee for the Care and Use of Animals.

Data analysis

For concentration-dependent studies in cell cultures, the data were best fit to the equation: carnosine uptake $= V_{max}$? C/(K_m + C) + K_d ? C, where V_{max} is the maximal rate of saturable carnosine uptake, K_m is the Michaelis constant, C is the substrate (carnosine) concentration, and K_d is the non-saturable rate constant. To evaluate if more than one class of transporters was operational for carnosine, a Woolf-Augustinsson-Hofstee transformation of the saturable portion of uptake (V = carnosine uptake – K_d ? C) was performed in which: $V = V_{max} - K_m$? V/C.

Statistical comparisons were performed with ANOVA (SYSTAT, version 8.0; SPSS Inc., Chicago, IL, USA) and pairwise comparisons were made with Dunnett's test. A probability of $p = 0.05$ was considered statistically significant. Linear and non-linear regression analyses were performed with SCIENTIST (version 2.01; MicroMath Scientific Software, Salt Lake City, UT, USA) and a weighting factor of unity. The quality of fit was determined by evaluating the coefficient of determination (r^2), the standard error of parameter estimates, and by visual inspection of the residuals. Data are reported as mean ± SE.

Results

Apical versus basolateral uptake and efflux of carnosine in cell cultures

The time course of [^{3}H]carnosine uptake in normal-Na^+ aCSF is shown in Fig. 1. The uptake rate was 5–6 times

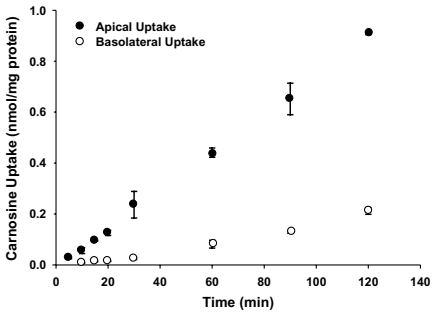

Fig. 1 Uptake amount of [^{3}H]carnosine as a function of time from cell monolayers (1 μM carnosine in external medium). (●) Uptake from the apical chamber; (○) uptake from the basolateral chamber. Uptake is linear through 120 min from both the apical and basolateral sides. Studies were performed using normal-Na^+ aCSF buffer, pH 7.4, in both chambers. Data are expressed as mean ± SE (n = 3–6).

greater from the apical than from the basolateral surface. The apical uptake was linear through 120 min, at which time there was approximately a 135 : 1 ratio of intracellular to extracellular carnosine, indicating active accumulation from CSF into the cell. Based on these results, a 60-min incubation time was chosen for initial rate uptakes of carnosine in order to maximize its sensitivity for kinetic and amino acid/dipeptide inhibitor studies. Alternatively, a 15-min incubation time was chosen for carnosine studies with charged compounds (i.e. organic anion or cation inhibitors) so that potential intracellular and/or extracellular pH changes would be minimized. Stability was not an issue in our experimental system as ≥99.3% of carnosine was recovered intact from the apical, basolateral and intracellular compartments, even after 2 hours of incubation.

To probe the fate of carnosine once in the cell, efflux was examined after a 2-h pre-loading period. As shown in Fig. 2,

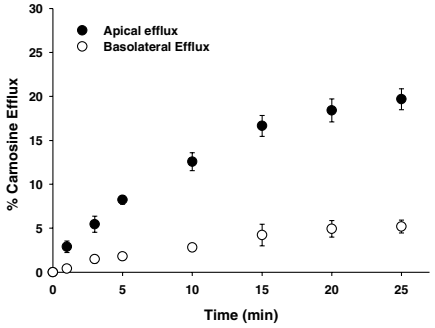

Fig. 2 Efflux of [^{3}H]carnosine as a function of time from cell monolayers (after loading for 2 h with 1 μM [^{3}H]carnosine). (●) Efflux into the apical chamber; (○) efflux into the basolateral chamber. Data reported as per cent of total carnosine effluxed at a given time point. Studies were performed using normal-Na^+ aCSF buffer, pH 7.4, in both chambers. Data are expressed as mean ± SE (n = 3–6).

at 25 min, about 20% of [^{3}H]carnosine was effluxed to the apical chamber while only 5% was effluxed to the basolateral chamber. The apical efflux was similar to values determined previously for GlySar in rat choroid plexus primary cell cultures (Shu *et al*. 2002). Although the uptake of carnosine occurred primarily at the apical membrane, its apical to basal transport could not be discerned from that of mannitol alone (data not shown). The lack of measurable transepithelial transport agrees with very minor efflux of carnosine into the basolateral compartment. Due to the dominance of carnosine's apical uptake, along with PEPT2 being localized to the apical surface of choroid plexus epithelial cells, the specificity and kinetic properties of carnosine were characterized further at this membrane surface alone.

Apical uptake of carnosine ± inhibitors in cell cultures

The specificity of peptide-mediated transport was examined using physiologic normal-Na^+ aCSF versus low-Na^+ aCSF buffer and a wide variety of potential inhibitors. As shown in Fig. 3(a) (physiologic buffer), neither β-alanine nor L-histidine, the constituent amino acids of carnosine, were able to inhibit uptake. However, unlabeled carnosine as well as two dipeptides, GlyGln and GlySar, reduced [^{3}H]carnosine uptake to values that were 5–10% of control. A similar inhibition profile was observed when radiolabeled carnosine was studied under low sodium conditions (Fig. 3b). As observed in Fig. 4(a) (physiologic buffer), the uptake of carnosine was inhibited 30–40% by the organic anions SITS and GABA, but not by TCA or PAH. In contrast, there was no inhibition of carnosine by organic acids when studied in low sodium buffer (Fig. 4b). Carnosine was also inhibited 35–40% by the organic cations NMN and TEA, but not by choline (Fig. 5a, physiologic buffer). However, carnosine was not inhibited by organic cations when studied under low sodium conditions (Fig. 5b).

Apical uptake kinetics of carnosine in cell cultures

The inhibition studies suggested that carnosine may be transported across the apical membrane by sodium-independent (i.e. PEPT2) and sodium-dependent processes. In order to assure the specificity of kinetic parameters, the saturable uptake of carnosine was determined in low-Na^+ aCSF buffer. This buffer has been shown to be advantageous in studying the PEPT2-mediated transport of dipeptides in choroid plexus (Teuscher *et al*. 2000, 2001). As shown in Fig. 6, the apical uptake of carnosine was of high affinity in which K_m and V_{max} values were estimated at 34 ± 6 μM and 73 ± 5 pmol/mg protein/min, respectively. Moreover, K_d values were small (i.e. 0.063 ± 0.007 μL/mg protein/min) and, as a result, the carrier-mediated transport accounted for >97% of the total uptake under linear conditions. A Woolf-Augustinsson-Hofstee plot of the saturable data (Fig. 6, inset) was also linear (r^2 = 0.829),

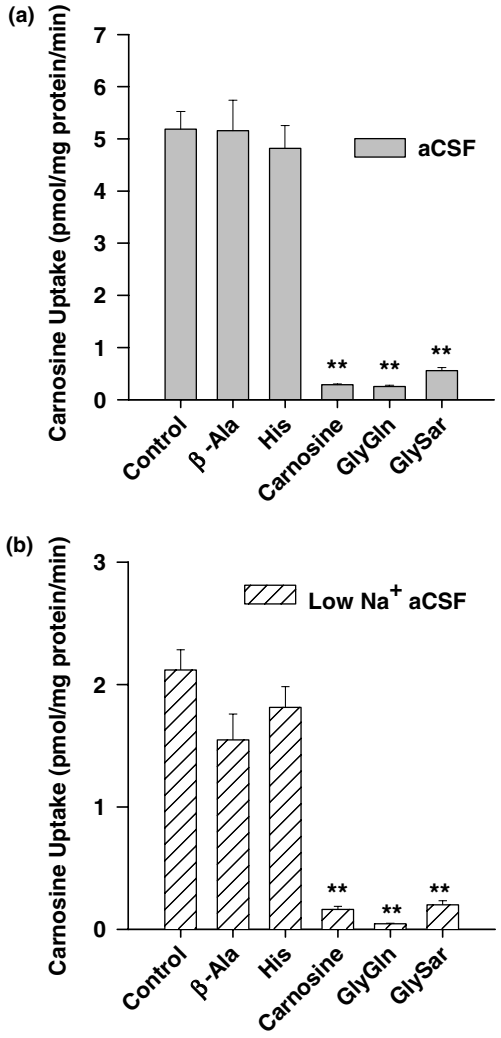

Fig. 3 Effect of amino acids or dipeptides on the apical uptake of [^{3}H]carnosine from cell monolayers (1 μM carnosine in external medium; inhibitor concentrations at 1 mM). Studies were performed using normal-Na^+ aCSF buffer, pH 7.4, in both chambers (a) or low-Na^+ aCSF buffer, pH 7.4, in both chambers (b). Data are expressed as mean ± SE (n = 6–10 for controls; n = 3–6 for inhibitors). **$p < 0.01$ as compared with control values.

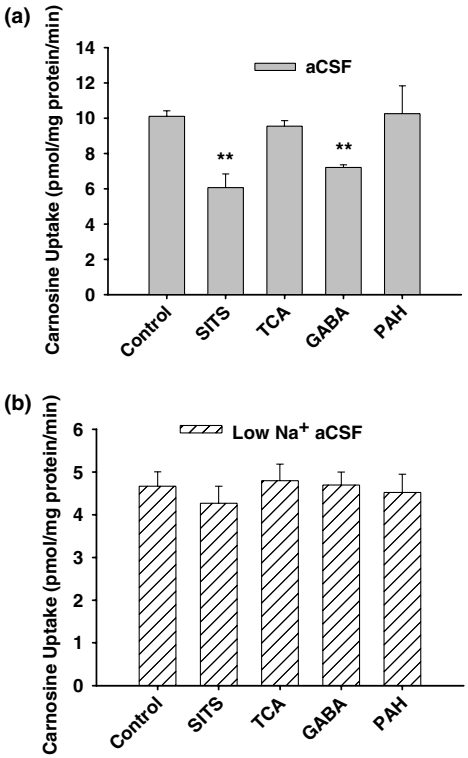

Fig. 4 Effect of organic anions on the apical uptake of [^{3}H]carnosine from cell monolayers (1 μM carnosine in external medium; inhibitor concentrations at 1 mM). Studies were performed using normal-Na^+ aCSF buffer, pH 7.4, in both chambers (a) or low-Na^+ aCSF buffer, pH 7.4, in both chambers (b). Data are expressed as mean ± SE (n = 6–10 for controls; n = 3–6 for inhibitors). **$p < 0.01$ as compared with control values.

indicating that a single transport system was functioning under low-Na^+ conditions.

Endogenous carnosine

Given the substantial accumulation of carnosine in choroid plexus, the *in vivo* concentrations of carnosine were determined in several tissues and body fluids of rats. The results in Table 1 demonstrate that there is a significant amount of carnosine stored in the choroid plexus (100 μmol/kg), as well as in the olfactory bulb (830 μmol/kg) and skeletal muscle (1.78 mmol/kg). In contrast, carnosine levels were below the limit of quantitation in CSF (< 2 μM) and plasma (< 0.4 μM).

Carnosine uptake in isolated choroid plexuses of PEPT2$^{+/+}$ and PEPT2$^{-/-}$ mice

The uptake of carnosine was evaluated at 2 min, a value that approximates initial rate uptake conditions in whole tissue choroid plexus. In the presence of physiologic normal-Na^+ aCSF buffer (Fig. 7), carnosine uptake was virtually eliminated in PEPT2$^{-/-}$ mice as compared with that in PEPT2$^{+/+}$ mice (V_d = 0.0436 μL/mg vs. 1.10 μL/mg, respectively; $p < 0.0001$). A similar reduction was observed using low-Na^+ aCSF buffer (V_d = 0.0481 μL/mg in PEPT2$^{-/-}$ mice vs. 0.675 μL/mg in PEPT2$^{+/+}$ mice; $p < 0.0001$). Under both conditions (i.e. normal and low sodium), the residual uptake of carnosine was ≤ 7% in PEPT2 null animals.

Discussion

The choroid plexus performs a number of essential functions in the brain. As the primary gatekeeper at the blood–cerebrospinal fluid barrier (BCSFB), the choroid plexus has a critical role in regulating the volume and composition of the interstitial fluid that surrounds the brain's neurons and

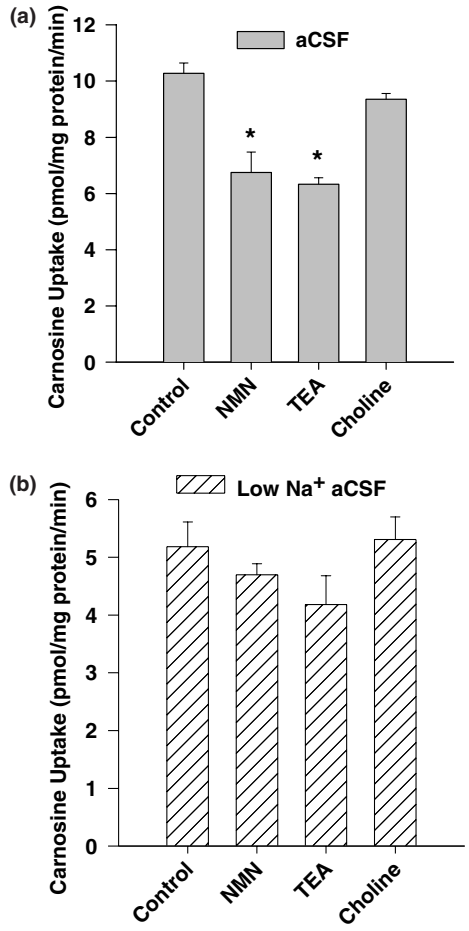

Fig. 5 Effect of organic cations on the apical uptake of [^{3}H]carnosine from cell monolayers (1 μM carnosine in external medium; inhibitor concentrations at 1 mM). Studies were performed using normal-Na^+ aCSF buffer, pH 7.4, in both chambers (a) or low-Na^+ aCSF buffer, pH 7.4, in both chambers (b). Data are expressed as mean ± SE (n = 6–10 for controls; n = 3–6 for inhibitors). $^{**}p < 0.01$ as compared with control values.

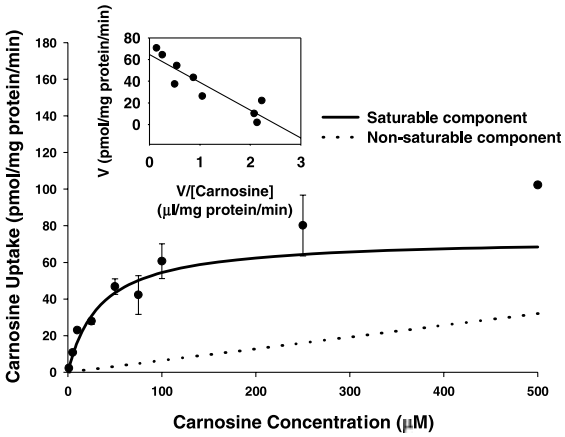

Fig. 6 Concentration-dependent apical uptake of [^{3}H]carnosine from cell monolayers (1–500 μM total carnosine in external medium). Studies were performed using low-Na^+ aCSF buffer, pH 7.4, in both chambers. The experimental data (•) are expressed as mean ± SE (n = 3–6). The predicted curves were generated using the V_{max}, K_m and K_d values, as determined by non-linear regression ($r^2 > 0.990$) and reported in the text. The insert is a Woolf-Augustinsson-Hofstee plot, which is linear ($r^2 = 0.829$). Error bars are omitted for clarity in the insert.

supportive glial cells. PEPT2 is expressed in choroid plexus, however, its physiological function is still unclear. Peptide transporters are known to serve a nutritive role in the intestine and as a mechanism for peptide conservation in the kidney (Leibach and Ganapathy 1996; Daniel and Herget 1997). Although PEPT2 may serve as a nutritive role by supplying peptides to the choroid plexus and CSF from blood, it more likely acts as a clearance mechanism to remove unwanted peptides or peptide fragments from the CSF. This belief is supported by studies on GlySar transport and directionality, and the apical localization of PEPT2 in choroid plexus (Teuscher *et al*. 2000; Shu *et al*. 2002). Because these papers focused on a synthetic dipeptide, there is little information on how endogenous peptides (and neuropeptides) are handled at the blood–CSF interface. Based on uptake studies in the absence and presence of sodium, preliminary studies have suggested that PEPT2 has a significant role in the choroid plexus uptake (about 30–60%) of 5-aminolevulinic acid (Novotny *et al*. 2000), GlySar (Teuscher *et al*. 2000) and carnosine (Teuscher *et al*. 2001). However, a more recent study in PEPT2 knockout mice (Shen *et al*. 2003) demonstrated that this transporter was responsible for about 90% of the uptake of GlySar in isolated choroid plexus under optimized conditions (i.e. Tris-MES buffer, pH 6.5). The current studies use a combination of rat choroid plexus primary cell cultures and transgenic mice to further probe the role of PEPT2 in neuropeptide homeostasis in the CSF under physiologic conditions.

We found that carnosine exhibited a preferential uptake at the apical membrane of the choroid plexus epithelial cells. However, there was no measurable transcellular transport of carnosine from the apical to basolateral chambers. This finding is supported by the low basolateral efflux, which measured only 5% at 25 min. Instead, it appears that once carnosine enters the choroid plexus from the CSF-facing side, it accumulates substantially within the cells, achieving concentrations 135 × higher than that of extracellular medium and *in vivo* levels of 100 μmol/kg. The culturing medium did not contain carnosine, which might suggest that our uptake studies demonstrate a replenishment of carnosine stores in various intracellular compartments. Our efflux results also indicate that some carnosine can recycle back to the CSF. The lack of carnosine transepithelial transport differs from studies by Shu *et al*. (2002) who demonstrated that GlySar was effluxed at the basolateral membrane. It is possible that carnosine and GlySar share a common uptake

Table 1 Endogenous levels of carnosine from adult rat tissues

Tissue	Concentration (mmol/kg) This Study[a]	Concentration (mmol/kg) Literature values[b]	References
Skeletal muscle	1.78 ± 0.03	0.7–1.36	(Chung-Hwang *et al.* 1976; O'Dowd *et al.* 1990; Ward and Preedy 1992)
Olfactory bulb	0.83 ± 0.01	0.9–2.7	(Margolis 1974; Ferriero and Margolis 1975)
Choroid plexus	0.10 ± 0.02	–	–
CSF	< LOQ	0	(Kish *et al.* 1979)
Plasma	< LOQ	–	–

[a]Data are reported as mean ± SE ($n = 3$). [b]Data are reported as range. [c]LOQ represents the limit of quantitation (0.002 mM for CSF and 0.0004 mM for plasma).

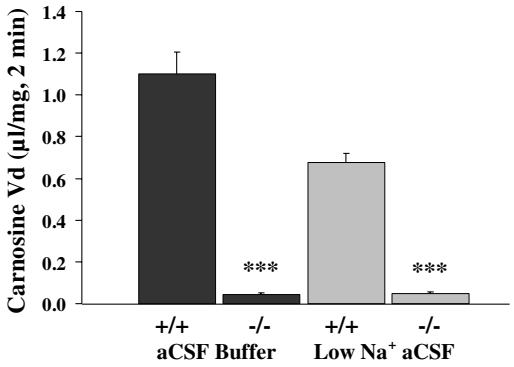

Fig. 7 Uptake of [^{3}H]carnosine in the isolated choroid plexuses of PEPT2$^{+/+}$ and PEPT2$^{-/-}$ mice (1 µM carnosine in external medium). Studies were performed using normal-Na^+ aCSF buffer, pH 7.4, or low-Na^+ aCSF buffer, pH 7.4, in both genotypes. Data are expressed as mean ± SE ($n = 3$–4). ***$p < 0.001$ as compared with wild-type animals.

pathway (i.e. PEPT2), but have different cellular efflux mechanisms at the basolateral membrane.

The apical uptake of carnosine was one of high affinity ($K_m = 34$ µM) and low capacity ($V_{max} = 73$ pmol/mg protein/min), and was consistent with a PEPT2-mediated process. In addition, the K_m determined for carnosine in this study (rat choroid plexus primary cell cultures) compared favorably with the K_m of 39.3 µM reported previously for carnosine in isolated choroid plexus experiments (Teuscher *et al.* 2001). The preferential and high-affinity uptake of carnosine further support the belief that PEPT2 serves as an efflux pump to clear peptides from the CSF. PEPT2 is well suited for this task as its K_m values are generally in the µM range for most di- and tripeptides. Working in concert with apically expressed peptidases in the choroid plexus (Smith *et al.* 2004), PEPT2 can effectively clear low concentrations of neuropeptides and/or their metabolites. A lack of inhibition by the constituent amino acids of carnosine (i.e. β-alanine and L-histidine), coupled with the stability of intact radiolabel, indicate that carnosine metabolism is not a confounding factor in these experiments. In addition, the peptide/histidine transporters, PHT1 and PHT2, do not appear to mediate carnosine uptake as demonstrated by the lack of inhibition by L-histidine at concentrations 50 times its K_m value (Yamashita *et al.* 1997) and because of results in the PEPT2 transgenic mice.

The studies in transgenic mice clearly demonstrate that PEPT2 accounts for over 90% of carnosine's uptake in choroid plexus whole tissue. The lack of difference in null animals as a function of sodium (normal-Na^+ vs. low-Na^+ aCSF buffer) suggests that residual sodium-dependent transport mechanisms are not present. Yet, under low sodium conditions, the uptake of carnosine was reduced by 39% in wild-type mice. In previous studies (Novotny *et al.* 2000; Teuscher *et al.* 2001), this finding would have been mistakenly interpreted as PEPT2 being responsible for 61% of carnosine's uptake with another 39% being handled by Na^+-dependent processes. As a result, one must use caution when interpreting data from different buffer conditions. Although PEPT2-mediated transport is proton-coupled, as opposed to being Na^+-coupled, it is a tertiary active process that depends on prior sodium and hydrogen ion gradients being established. To further probe this relationship, we studied the effect of a Na^+/H^+ exchange inhibitor, dimethyl amiloride (1 mM), on the apical uptake of [^{3}H]carnosine (1 µM) in rat choroid plexus primary cell cultures. Using normal-Na^+ aCSF buffer conditions, carnosine uptake was inhibited by 88% ($p < 0.01$ as compared with control values), demonstrating the profound influence of Na^+/H^+ exchange on the PEPT2-mediated transport of a dipeptide.

Although speculative, the minor inhibition observed by some organic anions (i.e. SITS and GABA, but not TCA and PAH) and cations (i.e. NMN and TEA, but not choline) probably reflects alterations in membrane potential and/or microclimate pH, thereby resulting in reduced uptake of carnosine in choroid plexus. It is highly unlikely that other transporters are involved because, as shown in this study, PEPT2 appears to account for essentially all of carnosine's transport into choroid plexus. As shown with GlySar (Shen *et al.* 2003) and cefadroxil (Ocheltree *et al.* 2004) in

transgenic mice, only 5% of choroid plexus uptake occurs, in general, by non-specific processes.

In conclusion, these studies are unique in first demonstrating the preferential apical uptake of an endogenous neuropeptide at the BCSFB, and the importance of PEPT2 in this process. These findings establish that PEPT2 is responsible for over 90% of carnosine's choroid plexus uptake, and suggest a critical role for PEPT2 in neuropeptide homeostasis in the central nervous system. Although PEPT2 transports a wide range of di- and tripeptides, peptide disposition may vary once in the cell. Thus, some peptides may be accumulated in the choroid plexus, as observed in this study for carnosine. Others may be effluxed into blood, as found for GlySar (Shu *et al*. 2002). Yet others may be metabolized intracellularly by the choroid plexus and then recycled. In order to more completely understand the role of PEPT2 in the brain, our laboratory is currently investigating the *in vivo* pharmacokinetics and tissue distribution of peptides/mimetics in wild-type and PEPT2 knockout mice.

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