PEPT2-Mediated Uptake of Neuropeptides in Rat Choroid Plexus

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Purpose. The peptide transporter PEPT2 was recently shown to be functionally active in rat choroid plexus, suggesting that it may play a role in neuropeptide homeostasis in the cerebrospinal fluid. This study, therefore, examined the role of PEPT2 in mediating neuropeptide uptake into choroid plexus.

Methods. Whole-tissue rat choroid plexus uptake studies were performed on GlySar in the absence and presence of neuropeptides and on carnosine.

Results. The neuropeptides NAAG, CysGly, GlyGln, kyotorphin, and carnosine inhibited the uptake of radiolabeled GlySar at 1.0 mM concentrations. In contrast, TRH, [D-Arg^2]-kyotorphin, glutathione, and homocarnosine did not inhibit GlySar uptake. Kyotorphin, an analgesic, was a competitive inhibitor of GlySar with a Ki of 8.0 μM. The direct uptake of carnosine was also shown to be mediated by PEPT2 in isolated choroid plexus (Km = 39.3 μM; Vmax = 73.9 pmol/mg/min). Radiolabeled carnosine uptake was inhibited by 1.0 mM concentrations of GlySar or carnosine but not homocarnosine, L-histidine, or β-alanine.

Conclusions. These findings indicate that PEPT2 mediates the uptake of a diverse group of neuropeptides in choroid plexus, and suggests a role for PEPT2 in the regulation of neuropeptides, peptide fragments, and peptidomimetics in cerebrospinal fluid.

KEY WORDS: PEPT2; GlySar; carnosine; neuropeptides; choroid plexus.

INTRODUCTION

PEPT2, a member of the proton-oligopeptide transporter family, is a Na^+-independent symporter that moves peptides and protons across biological membranes. It is considered to be of high affinity and low capacity with a Km in the micromolar region, and is primarily located in the kidney, lung, and brain (1–3). Recently, PEPT2 has been localized in choroid plexus by mRNA analysis (4) and immunoblotting (5). The peptide-histidine transporter PHT1, which transports histidine and small peptides with high affinity and in a proton gradient-dependent manner, has also been localized in choroid plexus (6). These two transporters show little homology (< 20% amino acid identity), but seem to have overlapping substrate specificity. Despite the similarities in substrate specificity, PEPT2, but not PHT1, is involved in the uptake of glycylsarcosine (GlySar), a hydrolysis and peptidase resistant dipeptide in choroid plexus (7).

The choroid plexus is known to control the contents of the cerebrospinal fluid (CSF) via multiple transport systems (8). This homeostatic control ensures that the CSF maintains appropriate ionic composition, nutrient content, and pH (8,9). Our laboratory recently demonstrated that the high-affinity peptide transporter, PEPT2, is expressed and functionally active in rat choroid plexus tissue, while PEPT1 is absent (5,7). The presence of this peptide transporter in choroid plexus raises questions as to its physiologic purpose at the blood-CSF barrier. Peptide transporters are known to serve as a nutritive transporter in the intestine and as a mechanism of peptide conservation in the kidney (2,10). Thus, PEPT2 in choroid plexus may serve a nutritive role by supplying peptides from the blood circulation to the choroid plexus and CSF. Alternatively, it may serve as a clearance mechanism to clear peptide fragments, neuropeptides, and peptidomimetics from the CSF.

Neuropeptides function as neurotransmitters, neuromodulators, and hormones in the central nervous system. We hypothesize that PEPT2 is involved in the homeostatic control of these peptides at the blood-CSF barrier located at the choroid plexus. As a first step to test this hypothesis, several neuropeptides (Fig. 1) were evaluated as potential inhibitors of GlySar uptake in isolated rat choroid plexus or as substrates for PEPT2 in this tissue. They include carnosine, cystylglycine (CysGly), glutathione (GSH), gylcylglutamine (GlyGln), homocarnosine, N-acetylaspartylglutamate (NAAG), thyrotropin releasing hormone (TRH), kyotorphin (KTP), and one structural analog, [D-Arg^2]-kyotorphin (DKTP). These compounds were selected because they are all di- or tripeptides, and they have physiological and pharmacological relevance in the brain. Understanding the homeostasis of these peptides and mimetics could have important implications for the treatment of CNS disorders and for providing new strategies in drug design, delivery, and targeting to the brain.

With this in mind, the objective of this study is to determine the relative affinity of a diverse group of neuropeptides for PEPT2 using the *in vitro* method of isolated rat choroid plexus uptake. This study also evaluates the inhibitory mechanism of KTP and the direct transport of a neuropeptide, carnosine, by PEPT2 in whole tissue choroid plexus.

MATERIALS AND METHODS

Materials

[^{14}C]GlySar (119 mCi/mmol) was purchased from Amersham (Chicago, IL) and [^{3}H]mannitol (19.9 Ci/mmol) from New England Nuclear Life Science Products (Boston, MA). [^{3}H]Carnosine (9 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA) and [^{14}C]mannitol (53 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Amino acids (β-alanine and L-histidine), NAAG, TRH, CysGly, GlyGln, KTP, DKTP, GSH, L-carnosine, and L-homocarnosine were purchased from Sigma (St. Louis, MO). Nitex nylon monofilament screening fabric

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ABBREVIATIONS: GlySar, glycylsarcosine; CysGly, cystylglycine; GSH, glutathione; GlyGln, glycylglutamine; NAAG, N-acetylaspartylglutamate; TRH, thyrotropin releasing hormone; KTP, kyotorphin; DKTP, [D-Arg^2]-kyotorphin; aCSF, artificial cerebrospinal fluid; CSF, cerebrospinal fluid; MES, 2-(N-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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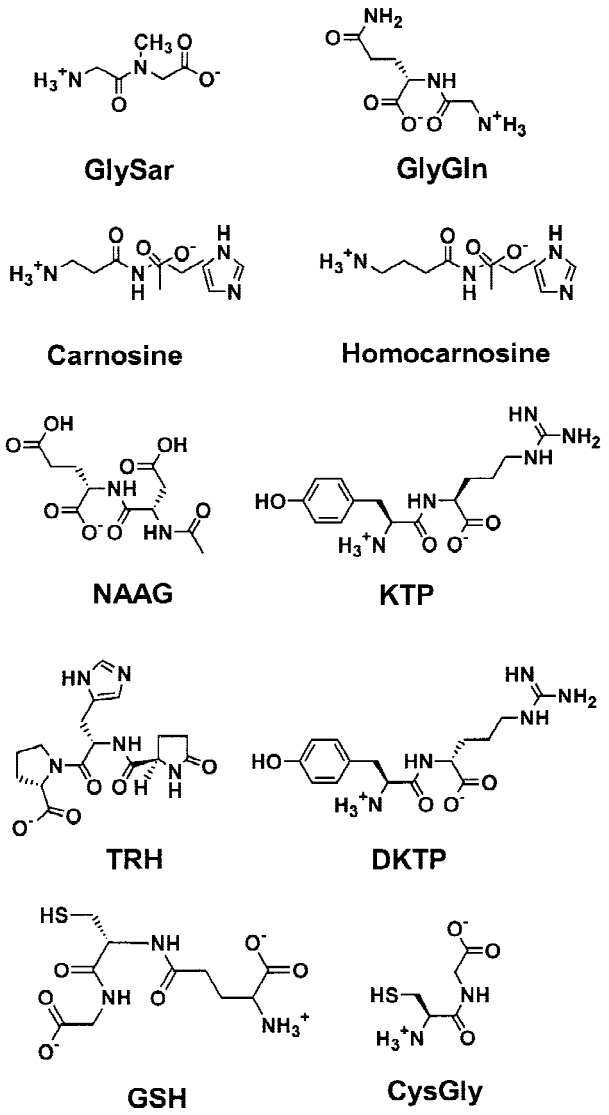

Fig. 1. Structures of neuropeptides evaluated in this study. Abbreviations defined in text.

(118 micron mesh opening) was purchased from Sefar America Inc. (Kansas City, MO). Other chemicals were obtained from standard sources and were of the highest quality available.

Buffers

Bicarbonate artificial cerebrospinal fluid (aCSF) and Tris-MES buffers were used in the tissue preparation and uptake experiments. The bicarbonate aCSF buffer (≈305 mOsm/kg) was continuously bubbled with 5% CO_2, 95% O_2, and 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 (pH 7.3). In experiments where a low sodium buffer was used, NaCl and $NaHCO_3$ were replaced by choline chloride and choline bicarbonate, respectively, producing a 1.0 mM sodium solution due to the presence of Na_2SO_4. The low sodium (1.0 mM) Tris-MES buffer (≈315 mOsm/kg) was continuously bubbled with 100% O_2 and contained 147mM choline chloride, 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, 5.0 mM glucose, and 10 mM Tris and/or MES. The pH of the Tris-MES buffer was adjusted to 6.5 using a combination of Tris and MES, while holding the osmolarity constant. A pH of 6.5 was previously determined to be the optimum value for PEPT2-mediated uptake in isolated choroid plexus tissue (7).

GlySar and Carnosine Uptake

Lateral ventricle choroid plexuses were isolated from anesthetized (pentobarbital, 65 mg/kg i.p.) male Sprague-Dawley rats aged 30–50 days, as described previously (5,7). The lateral ventricle plexuses were weighed and transferred to aCSF buffer at 37°C. There was a 5 min recovery period prior to the beginning of any experiment. After the recovery period, the plexuses were transferred to 0.95 ml of uptake buffer with or without drug for 0.5 min. Uptake was initiated by the addition of 0.05 ml of the uptake buffer with approximately 0.1 μCi of [^{14}C]GlySar and 0.2 μCi of [^{3}H]mannitol (an extracellular marker) or 0.1 μCi of [^{14}C]mannitol and 0.2 μCi of [^{3}H]carnosine. Unless otherwise stated, GlySar uptake was terminated after 3 min and carnosine uptake was terminated after 1 min by transferring the plexus to ice-cold aCSF buffer and filtering under reduced pressure. The filters (118 μm mesh) 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 prior to the addition of scintillation cocktail (Cytoscint) and counting with a dual channel liquid scintillation counter (Beckman LS 6000SC; Fullerton, CA). All animal procedures and study protocols adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985).

The uptake of radiolabeled substrate (GlySar or carnosine) into choroid plexus, in μl/mg of wet tissue weight, was calculated according to the following (7)

$$\text{Substrate Uptake} = \frac{\text{St} - \text{Sf} - [(\text{Mt}-\text{Mf})] \cdot \text{ratio}}{\text{Smedia}} \qquad (1)$$

where St is the total substrate (GlySar or carnosine) concentration in the plexus plus filter, Sf is the filter binding of substrate, and Smedia is the concentration of substrate in the external media. The term (Mt–Mf) · ratio is a correction for extracellular space where Mt is the total mannitol concentration in the plexus plus filter, and Mf is the filter binding of mannitol. Multiplying the difference between these two parameters by the ratio of [^{14}C]GlySar to [^{3}H]mannitol or [^{3}H]carnosine to [^{14}C]mannitol in the external medium provides an estimate of the extracellular content of GlySar or carnosine, respectively. The unidirectional influx rate (V) can then be calculated by multiplying Substrate Uptake by Smedia and dividing by duration of the experiment. Filter binding was <1% of the total uptake, and the extracellular space correction was 3–5%. Choroid plexuses were not perfused with saline prior to use and, therefore, some red blood cells are present in our tissue preparation. However, red blood cells do not significantly affect substrate uptake, as demonstrated previously (7).

Data Analysis

For the dose-response studies in which GlySar uptake can be completely abolished, the inhibitory effect is described by the model:

$$E = Eo \cdot \left(\frac{IC50^n}{IC50^n + I^n} \right) \quad (2)$$

where E is the observed uptake, Eo is the uptake in the absence of inhibitor, I is the inhibitor concentration, IC50 is the inhibitor concentration that causes 50% inhibition of maximal drug effect, and n is the slope factor. The parameters, IC50 and n, were estimated for each inhibitory neuropeptide by fitting the data to Eq. 2 using nonlinear regression.

KTP inhibited the uptake of GlySar (V) in a competitive manner (see Results section) and, as a result the kinetics become

$$V = \frac{Vmax \cdot C}{Km \cdot \left(1 + \frac{I}{Ki}\right) + C} \quad (3)$$

where Vmax is the maximal rate of GlySar uptake, Km is the Michaelis constant, Ki is the inhibition constant, and C is the substrate (GlySar) concentration. A Dixon transformation of Eq. 3 gives

$$\frac{1}{V} = \frac{Km}{Vmax \cdot Ki \cdot C} \cdot I + \frac{1}{Vmax} \cdot \left(1 + \frac{Km}{C}\right) \quad (4)$$

By plotting 1/V vs. inhibitor concentrations (I), a series of lines with slopes of Km/(Vmax · Ki · C) will be obtained. A further plot of the slopes of the Dixon plot vs. reciprocal substrate concentrations (1/C) will result in a line that passes through the origin. From the slope of this line (and knowing the Vmax and Km values), Ki can be estimated.

For kinetic studies of carnosine, the concentration-dependent uptake of substrate (V) was fit to the Michaelis-Menten relationship:

$$V = \frac{Vmax \cdot C}{Km + C} + Kd \cdot C \quad (5)$$

where Vmax is the maximal rate of carnosine uptake, Km is the Michaelis constant, Kd is the rate constant for nonsaturable transport, and C is the substrate (carnosine) concentration. To evaluate if more than one class of transporters was operational for carnosine, a Woolf-Augustinsson-Hofstee transformation of the saturable portion of carnosine uptake was performed

$$V = Vmax - Km \cdot \frac{V}{C} \quad (6)$$

Statistical comparisons were performed using analysis of variance (ANOVA; SYSTAT v8.0, SPSS Inc., Chicago, IL), and pairwise comparisons were made using Tukey's test. A probability of $P \leq 0.05$ was considered statistically significant. Linear and nonlinear regression analysis were performed using SCIENTIST (v2.01, MicroMath Scientific Software, Salt Lake City, UT) 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, unless otherwise indicated.

RESULTS

Inhibition of GlySar Uptake

Previous work in our laboratory demonstrated that dipeptide/mimetic uptake in the choroid plexus is mediated by two transport systems (5,7). PEPT2-mediated uptake represents the Na^+-independent portion of transport, which can be isolated and optimized in the choroid plexus by buffer manipulations (i.e., using low-sodium Tris-MES buffer at pH 6.5). Still, under more physiologic conditions using a bicarbonate-based CSF buffer, pH 7.3 (5,7), PEPT2-mediated transport represents a significant portion of the total uptake of GlySar (≥30%) and 5-aminolevulinic acid (≥50%). Therefore, to evaluate specific interactions between PEPT2 and selected neuropeptides (Fig. 1), a low-sodium Tris-MES, pH 6.5 buffer was used for the inhibition studies. NAAG, CysGly, GlyGln, KTP, and carnosine at 1.0 mM concentration all inhibit GlySar uptake (Fig. 2). Yet TRH, DKTP, GSH, and homocarnosine do not inhibit GlySar uptake. The dipeptides CysGly, GlyGln, KTP, and carnosine all inhibit 85–90% of GlySar uptake while NAAG, a modified dipeptide, only inhibits 50% of GlySar uptake. Based on these results, dose-response analyses were performed for NAAG, CysGly, GlyGln, KTP, and carnosine. Table I shows the IC50 values for each compound. NAAG has the highest IC50 value, about 0.6 mM, which compares well with only 50% inhibition at 1 mM concentration. The four remaining dipeptides have IC50 values ranging from 0.4 mM for carnosine to 5 μM for KTP.

The mechanism of inhibition for KTP was further examined because it had the lowest IC50 value, indicating that KTP is the most potent inhibitor in our group of neuropeptides. Figure 3 shows the Dixon plot of inhibition of GlySar uptake by KTP. Three lines are generated corresponding to three different concentrations of GlySar. These lines intersect above the *x*-axis, indicating a Ki of 7.1 μM (represents the median value). Replotting the slopes of the Dixon plot against the inverse GlySar substrate concentrations is shown

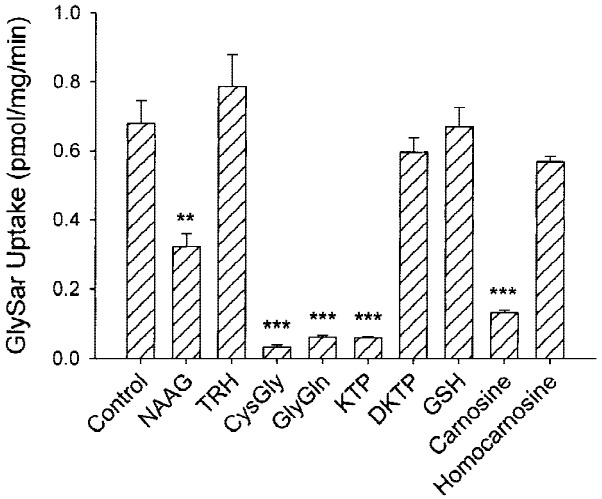

Fig. 2. Effect of selected neuropeptides on the uptake rate of [^{14}C]-GlySar (0.94 μM GlySar in external media). Inhibitor concentrations are 1.0 mM. Studies were performed using low-sodium Tris-MES CSF buffer, pH 6.5. Data are expressed as mean ± SE ($n = 8$ for control; $n = 4$ for inhibitors). ** $p < 0.01$, *** $p < 0.001$, as compared to control.

Table I. Dose-Response Analysis of Neuropeptide Inhibitors[a]

Neuropeptide	IC50 (μM)[b]	r^2
NAAG	615 ± 218	0.966
Carnosine	423 ± 43	0.982
CysGly	139 ± 14	0.983
GlyGln	14.1 ± 2.1	0.998
KTP	5.2 ± 1.3	0.997

[a] Inhibition of [^{14}C]GlySar (1.88 μM GlySar in external media) by the neuropeptides was fit to the model described in Eq. 2 (see text). IC50 is the concentration of inhibitor that causes a 50% reduction in GlySar uptake, and r^2 is the coefficient of determination for the fitted model. Inhibitor concentrations ranged from 0–10 mM for NAAG, 0–1.0 mM for carnosine, 0–1000 μM for CysGly, 0–500 μM for GlyGln, and 0–500 μM for KTP. Studies were performed in low-sodium Tris-MES buffer, pH 6.5.

[b] Results are expressed as mean ± SE (n = 4).

in the insert of Fig. 3, in which a line fit to the data points runs through the origin. This replot indicates that KTP is a competitive inhibitor of GlySar. From the slope of the line, and given the Km and Vmax of GlySar, the Ki is estimated by this method as 11.9 μM. The Ki may also be estimated by the method of Cheng and Prusoff (11) in which Ki is equivalent to IC50/(1 + C/Km) for a competitive inhibitor. The Ki is thus estimated to be 5.1 μM. From these three determinations, the Ki of KTP can be best estimated as 8.0 μM.

Carnosine Uptake

Because two transporters are known to transport dipeptides/mimetics in choroid plexus (5,7), we initially examined the uptake of carnosine (0.022 μM [^{3}H]carnosine in external media) in both aCSF and low-sodium aCSF buffers. Carnosine uptake in the more physiologic aCSF buffer (representing both Na^+-independent and Na^+-dependent mechanisms) was 0.0186 pmol/mg/min. In contrast, carnosine uptake in low-sodium aCSF (representing the Na^+-independent, PEPT2 mechanism) was 0.0112 pmol/mg/min. Thus, PEPT2-mediated transport of carnosine represents 60% of the total uptake. This, however, might be a minimal estimate of the contribution of PEPT2 because the low-sodium aCSF buffer can affect the Na^+/H^+ exchanger (8,9) which, in turn, can affect PEPT2 activity by reducing the electrochemical proton gradient. Regardless, a significant portion of carnosine transport is mediated by PEPT2 under physiologic conditions in choroid plexus.

To further evaluate the specific interaction between carnosine and PEPT2, studies with [^{3}H]carnosine were subsequently performed in low-sodium Tris-MES buffer at pH 6.5. Figure 4 shows the time-dependent uptake of [^{3}H]carnosine in rat choroid plexus. Uptake is linear for the first 2 min, thus we chose 1 min for the uptake time in our subsequent experiments. To verify that transport was specific for the PEPT2 transporter, we inhibited [^{3}H]carnosine uptake with 1 mM concentration of carnosine, GlySar, homocarnosine, L-histidine, and β-alanine (Fig. 5). Only carnosine and GlySar inhibited [^{3}H]carnosine uptake. We also evaluated the concentration-dependent uptake of [^{3}H]carnosine in choroid plexus tissue in which the data were fitted to a model containing one saturable component and one nonsaturable component (Fig. 6). Based on that model (Eq. 5), we determined the Km as 39.3 ± 14.0 μM, the Vmax as 73.9 ± 10.3 pmol/mg/min, and the nonsaturable component Kd as 0.044 ± 0.008 μl/mg/min. Because PEPT1 is not present in choroid plexus and the absence of sodium inactivates organic anion transport (5,7), the nonsaturable component seems to be either diffusion or an impurity in the radiolabel. The insert in Fig. 6 represents a Woolf-Augustinson-Hofstee transformation, which is linear (r^2 = 0.893), indicating a single class of transporters. Thus, carnosine is transported by a high-affinity, low-capacity transporter, which has the characteristics of PEPT2.

DISCUSSION

A diverse group of 9 neuropeptides were tested as inhibitors against the PEPT2-mediated uptake of GlySar in choroid plexus. Our results demonstrate that choroidal PEPT2 has significant affinity for some of these neuropeptides, and GlySar is inhibited by the neuropeptide kyotorphin

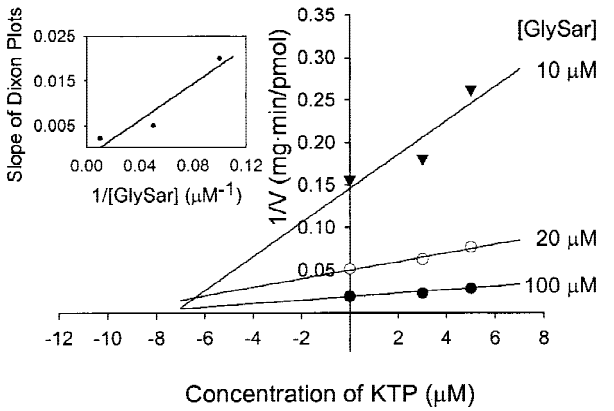

Fig. 3. Dixon plot of inhibition of [^{14}C]GlySar by KTP. Studies were performed using low-sodium Tris-MES CSF buffer, pH 6.5. Data are expressed as mean (n = 4). Lines generated by least squares regression. Insert is a replot of Dixon plot slopes vs. the reciprocal of the GlySar concentration. Regression is linear (r^2 = 0.911) with y-intercept not significantly different than zero (p = 0.728), indicating that KTP is a competitive inhibitor of GlySar.

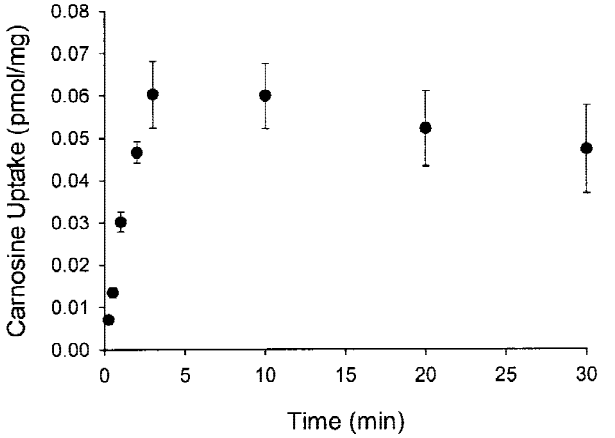

Fig. 4. Uptake amount of [^{3}H]carnosine as a function of time in rat lateral ventricle choroid plexus (0.022 μM carnosine in external media). Uptake is linear through 2 min, with y-intercept not significantly different that zero (P = 0.189). Studies were performed in low-sodium Tris-MES CSF buffer, pH 6.5. Data are expressed as mean ± SE (n = 4).

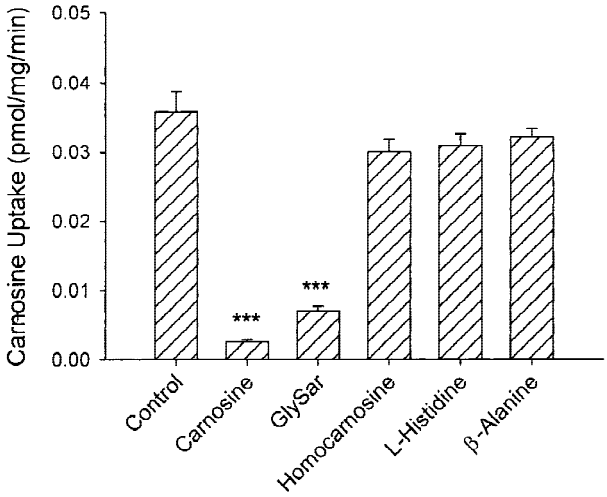

Fig. 5. Effect of selected inhibitors on the uptake rate of [^{3}H]carnosine (0.022 μM carnosine in external media). Inhibitor concentrations are 1.0 mM. Studies were performed using low-sodium Tris-MES CSF buffer, pH 6.5. Data are expressed as mean ± SE ($n = 8$ for control; $n = 4$ for inhibitors). *** $P < 0.001$, as compared to control.

in a competitive manner. We also demonstrate that carnosine uptake is mediated in part by choroid plexus PEPT2. Taken as a whole, these results suggest that PEPT2 plays an important role in neuropeptide homeostasis at the choroid plexus.

Only 5 of the 9 neuropeptides inhibited GlySar uptake, namely NAAG, CysGly, GlyGln, KTP, and carnosine. In contrast, TRH, homocarnosine, GSH, and DKTP did not inhibit GlySar uptake. TRH does not contain a free amino group because of the cyclization of glutamine to pyroglutamine. Experiments in sheep choroid plexus showed no TRH transport (12) and Fujita *et al.* (13) showed no interaction between TRH and PEPT2 in synaptosomes. Homocarnosine is similar to carnosine except it has an additional methyl group between the free amine and the carboxylic acid. According to Daniel *et al.* (14), a prescribed backbone length between two ionized

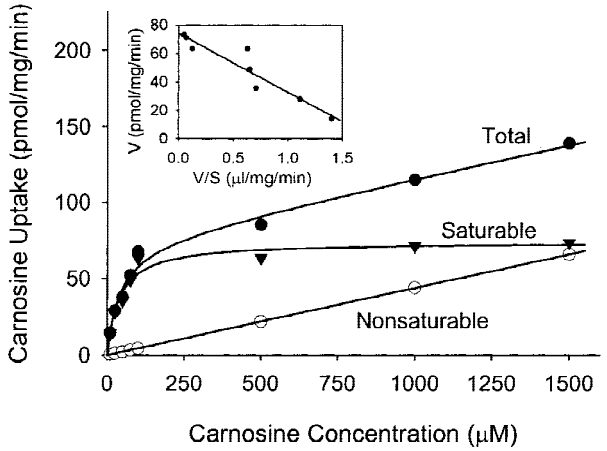

Fig. 6. Concentration-dependent uptake rate of [^{3}H]carnosine (10-1500 μM total carnosine in external media). Predicted curves are generated using Vmax, Km and Kd values reported in text ($r^2 \geq 0.987$). Insert is Woolf-Augustinsson-Hofstee plot, which is linear ($r^2 = 0.893$). Studies were performed using low-sodium Tris-MES CSF buffer, pH 6.5. Data are expressed as mean ± SE ($n = 4$). Error bars were omitted for clarity.

groups (i.e., amino and carboxyl group) is important for substrate recognition and transport by the intestinal peptide transporter. Thus, it seems that similar structural features are necessary for TRH or homocarnosine to interact with the renal peptide transporter PEPT2. GSH contains a highly reactive sulfhydryl group that is easily oxidized to the GSH-GSH dimer under physiological conditions. Although dimerization can be minimized under conditions of high acidity and low temperature, we were unable to pursue this option because of tissue viability. Thus, GSH may not show an effect in our system because it dimerizes to a hexapeptide that would not be transported. Further studies in a system that permits control of the dimerization process would help clarify if GSH is indeed a substrate of PEPT2.

DKTP is particularly interesting because it is a structural analog of KTP (D-arginine replaces L-arginine at the carboxy terminus). PEPT2 prefers L-amino acids (2), which could explain the difference in affinity for DKTP and KTP. DKTP has 6 times the analgesic effect of KTP (15), which can be attributed to reduced metabolism or clearance. If PEPT2 serves a clearance function in the CSF, then KTP would be cleared much faster that DKTP, thus allowing DKTP to accumulate and have greater pharmacological activity in the brain. Although this scenario is speculative, the relationship between transport properties and pharmacological effect in the central nervous system has already been shown in synaptic transmission of catecholamines (i.e., norepinephrine) (16).

The IC50 values for the 5 compounds that inhibit GlySar uptake vary from 5 to 615 μM. The weakest inhibitor, NAAG, is the most modified dipeptide with an extra acetyl group on the free amine, resulting in a secondary rather than primary amine. The next weakest inhibitor is carnosine, which contains a non-standard β-alanyl residue. The remaining three inhibitors (CysGly, GlyGln, and KTP) are all L-dipeptides with no modifications. All 3 dipeptides had IC50 values at or below the Km (129 μM) of GlySar, a modified dipeptide. Although all dipeptides are transported by PEPT2, their affinities for PEPT2 are quite different. A thorough examination of the effect of amino acid composition on dipeptide affinity for PEPT2 would be needed to draw further conclusions about the differences in IC50 values.

KTP was determined to be a competitive inhibitor of the PEPT2-mediated uptake of GlySar. The Ki of KTP in whole tissue choroid plexus was approximately 8.0 μM. This suggests that PEPT2 has high affinity for KTP, and that KTP is most likely transported by PEPT2. Human CSF levels of KTP are in the nM range (1.2 nM for healthy subjects, 0.24 nM for persistent pain subjects) (17,18). Thus, PEPT2 function could be involved in pain tolerance through regulation of KTP levels in the CSF. As alluded to above, DKTP may provide increased analgesia because of its reduced CSF clearance mediated by PEPT2. *In situ* analyses of KTP and DKTP transport and metabolism at the choroid plexus would be useful to further evaluate this premise. Finally, Fujita *et al.* (13) also demonstrated that KTP was a competitive inhibitor (Ki = 30 μM) of PEPT2-mediated GlySar uptake in synaptosomes prepared from rat cerebellum.

Carnosine uptake in kidney brush-border membrane vesicles has been demonstrated (19), however, the kinetic parameters of transport by PEPT2 have not been reported. In this study, we have shown that PEPT2 mediates carnosine uptake is isolated choroid plexus. We also demonstrated that

PEPT2-mediated carnosine uptake represents at least 60% of total uptake under physiologic conditions, and carnosine uptake is concentration-dependent. The Km is about 40 μM suggesting a high affinity transporter, and the Vmax is about 75 pmol/mg/min suggesting a low capacity transporter. Under linear conditions, about 98% of carnosine is transported by the saturable component. Carnosine uptake reached a plateau concentration by 4 min with a value of 0.055 pmol/mg. This corresponds to a tissue to media ratio of 2.5:1, suggesting that carnosine is actively accumulated in choroid plexus. Although carnosine has a relatively high IC50 value against GlySar, it has a faster initial uptake rate than GlySar (7).

To ensure that we were measuring carnosine uptake by PEPT2, we evaluated 5 potential inhibitors. Both carnosine and GlySar inhibited [^{3}H]carnosine uptake. In contrast, homocarnosine and the amino acid constituents of carnosine, L-histidine and β-alanine, did not inhibit [^{3}H]carnosine uptake. These results indicate that carnosine metabolism is not a confounding factor in the interpretation of our data. In addition, PHT1 does not appear to mediate carnosine uptake because of the lack of inhibition by L-histidine at concentrations 50 times the Km for L-histidine (6). These findings demonstrate that carnosine is being transported by PEPT2, and the process is specific.

In the kidney, PEPT2 works in concert with many peptidases that degrade large proteins into small di- and tripeptide fragments for reabsorption from the kidney lumen (3,20). It has been shown that many of the same peptidases present in kidney microvilli are present in choroid plexus brush border membranes (21). The presence of peptidases in the choroid plexus suggests that larger peptides are broken down in this tissue before being transported by PEPT2. Although the present study focused specifically on two- and three-amino-acid neuropeptides, larger neuropeptides may be digested by peptidases and then transported by PEPT2.

The regulation of choroid plexus and CSF pH is of particular interest because PEPT2-mediated peptide uptake is pH dependent in this tissue (7). Choroid plexus cell pH is affected by various factors including external pH, sodium, and bicarbonate concentrations (22,23). Additionally, pH regulation is compromised *in vitro* (22). Normally, bulk CSF pH is similar to that in plasma but choroid plexus epithelial cell pH is about 0.3 units less than bulk CSF (22). However, whether there are local pH gradients, for example at the choroidal apical membrane, is unknown (24). pH gradients do exist in the kidney and intestine, both of which exhibit proton-dependent peptide transport. In this regard, studies in intestinal and renal brush border membrane vesicles indicate that PEPT2-mediated transport is modulated by extracellular pH (19,25). Further, Wang *et al.* (24) demonstrated that external proton concentrations markedly affect brain-derived PEPT2 when expressed in Xenopus *laevis* oocytes.

Based on our findings, PEPT2 mediates a portion of neuropeptide transport in choroid plexus, suggesting that it might be involved in the biodistribution of peptidomimetics in the CSF and blood. According to Huang (26) in which dipeptide accumulation occurred on the apical side of the choroid plexus, we speculate that PEPT2 could be located on the CSF-facing membrane. Positioned there, PEPT2 could regulate the clearance of neuropeptides, unneeded peptide fragments, and peptidomimetics from the brain. Our data indicate that the PEPT2-mediated clearance of carnosine is 2 μl/min at physiologic pH, a value similar to CSF bulk flow in adult rats. However, this is only a rough estimate since we are comparing *in vitro* choroidal uptake versus *in vivo* CSF flow. Also, if the apical microenvironment pH, as observed in the small intestine (27) and presumably kidney, is more acidic then carnosine clearance would increase 3–5-fold. Understanding how PEPT2 affects the distribution of compounds across the choroid plexus barrier will help in future drug design and the more effective treatment of CNS disorders. For example, just as known P-glycoprotein inhibitors are given orally to promote P-glycoprotein substrate uptake in the intestine (28), so too could PEPT2 inhibitors be used to limit CSF clearance of desirable PEPT2 substrates.

In summary, our studies are unique in describing the interaction of a diverse group of neuropeptides with the proton/oligopeptide symporter, PEPT2, in isolated choroid plexus. We demonstrate, for the first time, the competitive inhibition of GlySar uptake by KTP as well as the direct transport of carnosine in this tissue. These findings suggest that choroidal PEPT2 plays a role in neuropeptide homeostasis of cerebrospinal fluid. Our laboratory is in the process of collecting immunocytochemical data regarding the precise cellular location of PEPT2 and developing choroid plexus epithelial cells in primary culture with the aim of studying the directionality of peptide/mimetic transport by PEPT2 (29).

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