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Divergent developmental expression and function of the proton-coupled oligopeptide transporters PepT2 and PhT1 in regional brain slices of mouse and rat

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Abstract

This study evaluated the developmental gene and protein expression of proton-coupled oligopeptide transporters (POTs: peptide transporter, PepT1 and PepT2; peptide-histidine transporter, PhT1 and PhT2) in different regions of rodent brain, and the age-dependent uptake of a POT substrate, glycylsarcosine (GlySar), in brain slices. Slices were obtained from cerebral cortex, cerebellum and hippocampus of wildtype and PepT2 null mice, and from rats at different ages. Gene and protein expression were determined by real-time PCR and immunoblot analyses. Brain slice uptakes of radiolabeled glycylsarcosine were determined in the absence and presence of excess unlabeled glycylsarcosine or L-histidine, the latter being an inhibitor of PhT1/2 but not PepT1/2. As PepT2 and

PhT1 transcripts were abundantly expressed in all three regions of mouse brain, little to no expression was observed for PepT1 and PhT2. PhT1 protein was present in brain regions of adult but not neonatal mice and expression levels increased with age in rats. Glycylsarcosine uptake, inhibition and transporter dominance did not show regional brain or species differences. However, there were clear age-related differences in functional activity, with PepT2 dominating in neonatal mice and rats, and PhT1 dominating in adult rodents. These developmental changes may markedly impact the neural activity of both endogenous and exogenous (drug) peptides/mimetics.

Keywords: brain, development, expression, function, PepT2, PhT1.

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The activity of small peptides and peptide-like drugs in brain is tightly regulated by mechanisms controlling their concentration in the interstitial fluid. Mechanisms may include the synthesis and release of peptides/mimetics from brain parenchyma, entry and/or efflux via the blood-brain and blood-cerebrospinal fluid barriers, degradation by membrane-bound or intracellular enzymes and uptake into glial and neuronal cells (Keep and Smith 2013). A number of studies have investigated the role of specific oligopeptide transporters in regulating peptide/mimetic transport in the brain and, in particular, at the apical interface of the blood-CSF barrier (i.e., CSF-facing side of the choroid plexus) (Smith *et al.* 2004). However, few studies have evaluated whether or not differences exist in the transporter-mediated disposition of peptides/mimetics in various regions of the brain (Smith *et al.* 2011).

The proton-coupled oligopeptide transporter (POT) family is phylogenetically conserved and its members serve as integral membrane proteins in the cellular uptake of di/tripeptides and some pharmacologically active drugs (Wang *et al.* 2010; Smith *et al.* 2013). This family consists of four mammalian carriers, of which three are expressed in brain. PepT2 (SLC15A2), a low-capacity high-affinity carrier, is

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significantly expressed in brain as well as kidney, lung and mammillary gland (Shen *et al.* 1999, 2004; Brandsch *et al.* 2008; Rubio-Aliaga and Daniel 2008). PhT1 (SLC15A4), a high-affinity carrier, is abundantly expressed in brain and eye (Yamashita *et al.* 1997). PhT2 (SLC15A3) is expressed mainly in the lymphatic system and only faintly in brain (Sakata *et al.* 2001). The remaining carrier, PepT1 (SLC15A1), transports peptides/mimetics in a high-capacity low-affinity manner and is almost exclusively expressed in small intestine (Walker *et al.* 1998; Groneberg *et al.* 2001; Jappar *et al.* 2010). In contrast to PepT2 and PepT1, both PhT1 and PhT2 transport histidine as well as oligopeptides (hence the designation as peptide-histidine transporters; PhTs).

Several studies have demonstrated that PepT2 significantly affects both the disposition and pharmacological response of peptides/mimetics in brain. PepT2 is localized at the apical surface of choroid plexus and, as such, can substantially alter the relationship between CSF and plasma concentrations (Shen *et al.* 2004). For example, using genetically modified mice, it was shown that the synthetic dipeptide glycylsarcosine (GlySar), the peptidomimetic heme precursor 5-aminolevulinic acid, and the β-lactam peptide-like antibiotic cefadroxil had CSF-to-plasma concentration ratios in *PepT2* null mice that were 4–8 times greater than in wildtype animals (Kamal *et al.* 2008). These dispositional differences translated into greater protection of wildtype mice against 5-aminolevulinic acid-induced neurotoxicity (Hu *et al.* 2007) as well as greater L-kyotorphin-induced analgesia during PepT2 ablation (Jiang *et al.* 2009).

Immunoblot analyses in rats demonstrated that PepT2 is found widely throughout the brain including regions such as the cerebral cortex, olfactory bulb, basal ganglia, cerebellum and hindbrain (Shen *et al.* 2004). Moreover, PepT2 is developmentally expressed. Protein levels in cerebral cortex are maximal in foetus and decline rapidly with age. Immunolocalization by double-labeling experiments indicated that PepT2 is expressed in astrocytes and neurons of neonatal rats, but only in neurons of adult animals. However, functional studies were not performed and, as a result, it is unclear if heterogeneity exists in the PepT2-mediated transport of peptides and peptide-like drugs in different brain regions during development. Moreover, almost nothing is known about the functional activity and developmental expression of the peptide/histidine transporter PhT1 in brain. With this in mind, we proposed the following aims: (i) to delineate the expression of SLC15 transcripts (i.e., PepT1, PepT2, PhT1 and PhT2) and protein (i.e., PhT1) in brain cortex, cerebellum and hippocampus of adult and neonatal wildtype and PepT2 null mice and (ii) to examine the uptake properties of GlySar, a POT substrate, in regional brain slices from these mice. Adult and neonatal rats were also studied for comparison. In doing so, a relationship could be established between SLC15 gene - protein expression and transporter activity as a function of brain region, age and rodent species.

Methods

Materials

[Glycine-U-^{14}C]GlySar (106 mCi/mmol; purity 97.3% was obtained from Amersham Life Science (Piscataway, NJ, USA) and [^{3}H] mannitol (20 mCi/mmol; purity 99.0%) from American Radiolabeled Chemicals (St Louis, MO, USA). Unlabeled GlySar and L-histidine were obtained from Sigma-Aldrich (St Louis, MO, USA) and hyamine hydroxide from MP Biomedicals (Irvine, CA, USA). All other chemicals were obtained from standard sources. Wildtype mice ($PepT2^{+/+}$) and null mice ($PepT2^{-/-}$) mice were generated in-house (Shen *et al.* 2003) in which gender-matched neonatal (day 2) and adult (week 8–10) animals were used for the proposed experiments. Late gestation Sprague-Dawley rats were purchased from Charles River Laboratories (Portage, MI, USA) from which gender-matched rat progeny (day 2 to week 8) were studied. The animals were fed a standard diet, had access to water *ad libitum*, and were maintained in a temperature-controlled environment of 12-h light and dark cycles. Animal studies were conducted in accordance with the guidelines from the National Institutes of Health for the care and use of animals, and were approved by the Institutional Animal Care and Use Committee at the University of Michigan.

Real-time PCR of POT gene expression in brain

Following anaesthesia with sodium pentobarbital (40–60 mg/kg ip), the cerebral cortex, cerebellum and hippocampus were removed from mouse and rat brains. Total RNA was extracted using the Qiagen RNeasy Mini Kit (Hamburg, Germany) followed by reverse transcription using the Qiagen Omniscript RT Kit (16.5 μL H$_2$O containing 4.0 μg total RNA, 2.0 μL 15-mer random primers, 4.0 μL 0.1 M dithiothreitol, 8.0 μL 10 mM dNTPs, 8.0 μL 5 × buffer, 0.5 μL Ranisin and 1.0 μL 200 units enzyme), according to the manufacturer's protocols. Real-time PCR was carried out on an Applied Biosystems 7300 Real-Time PCR System (Foster City, CA, USA) using TaqMan-based detection for mice (9.0 μL H$_2$O containing 100 ng cDNA, 12.5 μL Taqman master mix, 1.25 μL 10 μM forward and reverse primers each, 1.0 μL 5.0 μM probe) and SYBR Green-based detection for rats (7.5 μL H$_2$O containing 30 ng cDNA, 12.5 μL SYBR Green master mix, 2.5 μL 5 μM forward and reverse primers each) (Nolan *et al.* 2006). The primers, probes and standard DNAs were synthesized by Integrated DNA Technologies (Coralville, Iowa) as listed in Table 1. Real-time PCR conditions were one cycle at 50°C × 2 min, one cycle at 95°C × 10 min, 40 cycles at 95°C × 15 s and one cycle at 60°C × 1 min. The absolute amount of a POT in mice was calculated automatically based on the standard curve, and expressed as the ratio of the POT to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Relative mRNA levels of PhT1 in rats were calculated using the ΔCT method in which the ratio of PhT1 to GAPDH genes was equal to $2^{-\Delta CT}$, ΔCT = CT (PhT1) – CT (GAPDH).

Immunoblots of PhT1 protein expression in brain

Following sodium pentobarbital anesthesia (40–60 mg/kg ip), regional brain tissues from mice and rats were homogenized in

Table 1 PCR primers, probes and standards for Slc15 gene expression studies in mouse (m) and rat (r) brain

mPepT1	Forward primer	5′-CCACGGCCATTTACCATACG-3′
	Reverse primer	5′-TGCGATCAGAGCTCCAAGAA-3′
	Probe	5′-/56-FAM/-CGTTGCCCTCTGCTACCTGACTCCA-/36-TAMSp/-3′
	Standard	5′-GACAATCTCTCCACGGCCATTTACCATACGTTCGTTG CCCTCTGCTACCTGACTCCAATTCTTGGAGCTCTGATCGCAGACTCGTGGC-3′
mPepT2	Forward primer	5′-TGCAGAGGCACGGACTAGATAC-3′
	Reverse primer	5′-GGGTGTGATGAACGTAGAAATCAA-3′
	Probe	5′-/56-FAM/-TCTCGGTCTTCTACCTCTCCATCAATG CA-/36-TAMSp/-3′
	Standard	5′-AAGAGGAACATGCAGAGGCACGGACTAGATACTTCTC GGTCTTCTACCTCTCCATCAATGCAGGGAGCTTGATTTCTACGTTCATCACACCCATGCTAAG-3′
mPhT1	Forward primer	5′-GCTGCCACCTGCATTACTACTTC-3′
	Reverse primer	5′-CGTACTTCACAGACACAATGAGGAA-3′
	Probe	5′-/56-FAM/-CTGGCCGCCATCCAGGGAGC-/36-TAMSp/-3′
	Standard	5′-AACATCAACAGCTGCCACCTGCATTACTACTTCTTC CTGCTGGCCGCCATCCAGGGAGCTACGCTGCTGCTCTTCCTCATTGTGTCTGTGAAGTACGACCGCCAGCG -3′
mPhT2	Forward primer	5′-GCTGAAGCTTGCGTTCCAA-3′
	Reverse primer	5′-AACAGGTGGGCACTTTCAGAGT-3′
	Probe	5′-/56-FAM/-CTGCTGTCCCTGCCGGAAGTCCT-/36-TAMSp/-3′
	Standard	5′-TGTCATCTATGCTGAAGCTTGCGTTCCAAAACTGCTGTCCC TGCCGGAAGTCCTCTTCCAGGGACTCTGAAAGTGCCCACCTGTTGCCTGACCAGA-3′
mGAPDH	Forward primer	5′-GAGACAGCCGCATCTTCTTGT-3′
	Reverse primer	5′-CACACCGACCTTCACCATTTT-3′
	Probe	5′-/56-JOE/-CAGTGCCAGCCTCGTCCCGTAGA-/36-TAMSp/-3′
	Standard	5′-TCCCTGTTCCAGAGACAGCCGCATCTTCTTGTGCAGTGCCA GCCTCGTCCCGTAGACAAAATGGTGAAGGTCGGTGTGAACGGATTTG-3′
rPhT1	Forward primer	5′-GGTGTGGCCACGGTCAA-3′
	Reverse primer	5′-GGCCTCTGGACCTCGATCTT-3′
rGAPDH	Forward primer	5′-TGGCCTCCAAGGAGTAAGAAAC-3′
	Reverse primer	5′-GGCCTCTCTCTTGCTCTCAGTATC-3′

2.0 mL Nonidet P40-lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P40 and proteinase inhibitor cocktail, pH 8.0), sonicated for 10 pulses at one-half strength, and then centrifuged at 15 000 g × 15 min at 4°C. Protein concentrations were measured using the bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA), after which 20 μg protein samples were separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Following pre-incubation with Tris-buffered saline with Tween 20 (TBST) buffer containing 5% non-fat milk (~ 1 h), the primary antibody, rabbit anti-rat PhT1 antiserum (Sun *et al.* 2013), was added to the TBST buffer (1 : 1000 dilution) and incubated for 60 min at 24°C. The membranes were washed three times, the secondary antibody added, goat anti-rabbit IgG-horseradish peroxidase antiserum (1 : 3000 dilution) and then incubated for 45 min at 24°C. After washing four times with TBST buffer, the bound antibody was detected with Immobilon Western Chemiluminescent horseradish peroxidase substrate (Millipore, Billerica, MA, USA).

GlySar uptake in regional brain slices

Once anaesthetized with sodium pentobarbital (40–60 mg/kg ip), the mice and rats were decapitated and their cerebral cortex,

cerebellum and hippocampus dissected and weighed. Brain slices, 300 μm thick, were then prepared in artificial CSF (aCSF) using a McIlwain tissue chopper (Brinkmann Instrument, Westbury, NY, USA) (Reid *et al.* 1988) The aCSF buffer consisted of (mM) 127 NaCl, 20 NaHCO$_3$, 2.4 KCl, 0.5 KH$_2$PO$_4$, 1.1 CaCl$_2$, 0.85 MgCl$_2$, 0.5 Na$_2$SO$_4$ and 5.0 glucose (pH 7.4). During experimentation (see below), the aCSF buffer was warmed to 37°C and continuously bubbled with 5% CO$_2$ and 95% O$_2$.

Regional brain slices were pre-incubated for 10 min and then transferred into 0.5 mL fresh aCSF buffer containing 2.0 μM [^{14}C]GlySar (0.1 μCi) and [^{3}H]mannitol (0.2 μCi). For some studies, excess (5 mM) unlabeled GlySar or L-histidine was added to the incubation buffer containing radiolabeled GlySar. After a 10 min incubation period (determined by preliminary studies to be optimal), 1.0 mL ice-cold aCSF buffer was added to terminate the reaction. The sample was immediately filtered through 100 μm nylon mesh, under reduced pressure, and then washed four times with 1.0 mL ice-cold aCSF buffer. The filter and tissue slice were transferred to a scintillation vial, 0.33 mL hyamine hydroxide was added and the vial situated overnight at 24°C until the tissue slices had dissolved. A 7.0 mL aliquot of Cytoscint cocktail (MP Biomedicals, Solon, OH, USA) was mixed and the

radioactivity measured by a Beckman LS 6000 SC dual-channel liquid scintillation counter (Beckman Coulter Inc., Fullerton, CA, USA). Uptake of GlySar in the brain slice was expressed as (Teuscher *et al.* 2000):

$$\text{GlySar Uptake} = \frac{\left(\text{GlySar}_t - \text{GlySar}_f\right) - \left(\text{Mannitol}_t - \text{Mannitol}_f\right) \bullet \dfrac{\text{GlySar}_{\text{media}}}{\text{Mannitol}_{\text{media}}}}{\text{TissueWeight} \bullet \text{GlySar}_{\text{media}}}$$

in which the uptake (µL/g wet tissue weight) was corrected for both filter binding and extracellular content. GlySar_t and GlySar_f were the amounts of GlySar in the tissue plus filter and filter binding, respectively, Mannitol_t and Mannitol_f were the respective amounts of mannitol in the tissue plus filter and filter binding, and $\text{GlySar}_{\text{media}}$ and $\text{Mannitol}_{\text{media}}$ were the concentrations of GlySar and mannitol in the external media.

Data analysis and statistics

Dose-response parameters were evaluated in regional brain slices using the partial inhibition equation:

$$\text{Uptake} = \text{No Inh} - \frac{I_{\max} \bullet \text{Inh}}{IC_{50} + \text{Inh}}$$

where the uptake of substrate (GlySar) was determined as a function of increasing inhibitor concentrations. According to this relationship, No Inh represented the uptake of GlySar in the absence of L-histidine, $I_{\max}$ was the maximal inhibition of GlySar uptake by L-histidine, IC_{50} was the concentration of L-histidine at which one-half the $I_{\max}$ was reached, and *Inh* was the concentration of inhibitor (L-histidine). The relationship was evaluated by non-linear least-squares regression using Prism v5.0 (GraphPad Software, Inc., La Jolla, CA, USA) and a weighting of unity. Quality of the fit was evaluated by standard error of the parameters, the coefficient of determination (r^2) and by visual inspection of the residuals.

Data were reported as mean ± SE unless otherwise indicated. A two-tailed Student's *t* test was used to compare statistical differences between two groups. For multiple comparisons, one-way analysis of variance was used followed by Tukey's or Dunnett's test, as appropriate, for pairwise comparisons between the groups. A $p \leq 0.05$ was considered statistically significant.

Results

SLC15 gene – PhT1 protein expression and GlySar uptake in regional brain slices from adult mice

The relative gene expression of SLC15 family members in adult wildtype and PepT2 null mice is displayed in Fig. 1. The same expression pattern was evident in cerebral cortex (Fig. 1a), cerebellum (Fig. 1b) and hippocampus (Fig. 1c), in which PepT2 and PhT1 transcripts were abundantly expressed. In contrast, little to no gene expression of PepT1 and PhT2 was observed. In performing immunoblot analyses of PhT1 protein, the transporter was present in all three regions of the brain in adult wildtype and PepT2 null mice (Fig. 2). With respect to function, transport inhibitors had a

similar effect on the uptake of radiolabeled GlySar in cortical, cerebellar and hippocampal brain slices of adult mice (Fig. 3). In the presence of 5 mM unlabeled GlySar, the uptake of 2 µM [^{14}C]GlySar was reduced by about 75% and 62%, respectively, in wildtype and PepT2 null mice. The uptake of GlySar was reduced to less than 10% of control values by 5 mM L-histidine in both genotypes. In contrast, there was no significant difference in GlySar uptake between wildtype and PepT2 null mice in slices prepared from cerebral cortex (Fig. 3a) and cerebellum (Fig. 3b). A minor reduction (24%), however, was observed in the hippocampus of null animals (Fig. 3c). Collectively, the results suggest that PhT1, and not PepT2, has a major influence on GlySar uptake in regional brain slices from adult mice.

To further explore the role of PhT1 in mouse brain, we performed a dose-response inhibitory analysis of 2 µM GlySar uptake by 0.001 µM to 5 mM L-histidine in cortical, cerebellar and hippocampal brain slices from adult PepT2 null mice. As shown in Table 2 and Fig. 4, a dose-dependent reduction in GlySar uptake was observed in which the IC_{50} of L-histidine ranged from 0.76 µM in cerebral cortex to 1.25 µM in hippocampus. Moreover, about 80% of the GlySar uptake was maximally inhibited by L-histidine, regardless of brain region.

SLC15 gene – PhT1 protein expression and GlySar uptake in regional brain slices from neonatal mice

The relative gene expression of SLC15 family members in neonatal wildtype and PepT2 null mice is displayed in Fig. 5. These results were very similar to that of adult mice in that PepT2 and PhT1 were highly expressed in cerebral cortex (Fig. 5a), cerebellum (Fig. 5b) and hippocampus (Fig. 5c), whereas PepT1 and PhT2 were not expressed to any meaningful degree in these regions. One notable difference was that the expression of PepT2 and PhT1 mRNA was 3.2- and 2.4-fold greater in neonates than in adults, respectively, for all tissue slice preparations ($p < 0.01$). Moreover, immunoblot analyses clearly demonstrated that PhT1 protein was absent from all brain regions of neonatal wildtype and PepT2 null mice (Fig. 2). Functionally, 5 mM of unlabeled GlySar reduced the uptake of 2 µM [^{14}C]GlySar in neonatal brain slices from wildtype and PepT2 null mice by about 64% and 53%, respectively (Fig. 6). However, in contrast to adult mice (Fig. 3), the uptake of GlySar under control conditions was substantially reduced (> 50%) in neonatal PepT2 null when compared
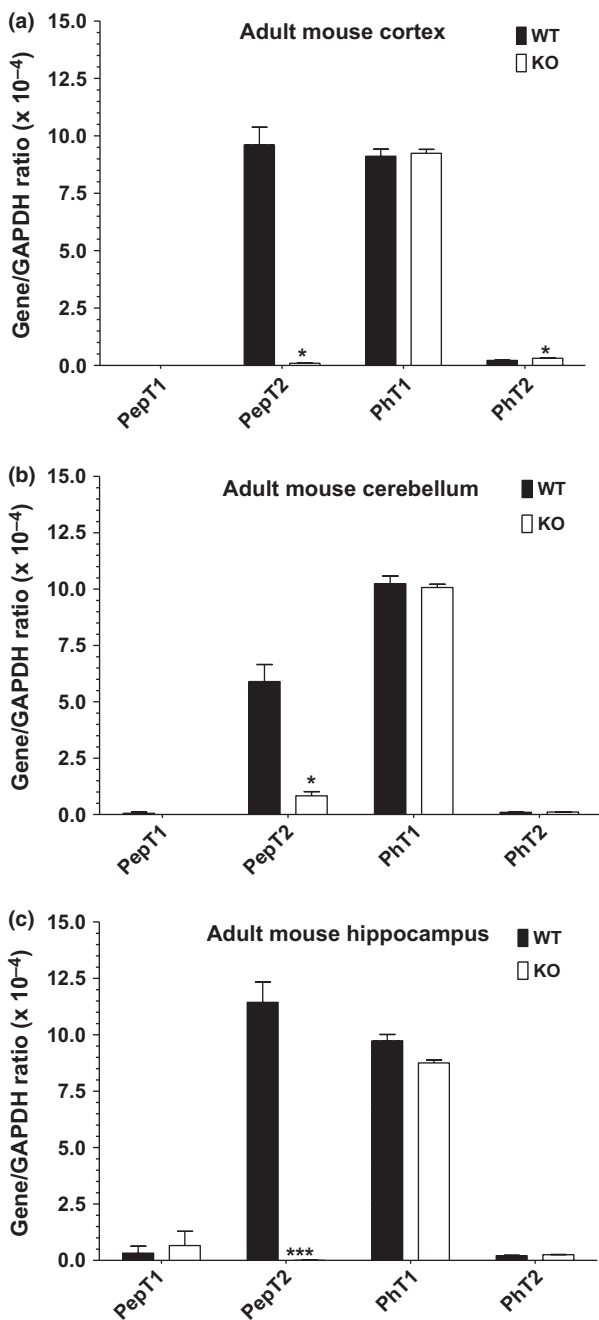
Fig. 1 Real-time PCR of proton-coupled oligopeptide transporter (POT) transcripts in the cerebral cortex (a), cerebellum (b) and hippocampus (c) of adult wildtype (WT) and PepT2 null (KO) mouse brain. Data are reported as the ratio of POT to GAPDH determined in each sample. Values are mean ± SE ($n = 3$). *$p \leq 0.05$ and ***$p \leq 0.001$ are compared with WT mice.

with wildtype mice for all three brain regions. In addition, neonatal mice did not exhibit an effect of L-histidine on GlySar uptake in cortical (Fig. 6a), cerebellar (Fig. 6b) and hippocampal (Fig. 6c) brain slices, regardless of genotype. Collectively, the results suggest that PepT2, and not PhT1,

has a major influence on GlySar uptake in regional brain slices from neonatal mice.

SLC15 gene – PhT1 protein expression and GlySar uptake in regional brain slices from developing rat

Given that peptide transporter expression and activity were typically performed in mice and/or rats, further studies were initiated in developing rat brains. As shown in Fig. 7a, PhT1 mRNA was present in the cerebral cortex, cerebellum and hippocampus of day 2 to adult rats with no consistent trend, although expression levels tended to be higher in neonatal and younger animals. Despite this observation, immunoblot analyses demonstrated that PhT1 protein expression was not observed for the first 7 days after birth but increased steadily in all three brain regions, reaching a maximal value in the adult rat (Fig. 7b). We also found that 5 mM unlabeled GlySar or L-histidine reduced the uptake of 2 μM [14C] GlySar by 63% and 81%, respectively, in the hippocampal region of adult rat brain (Fig. 7c). However, in neonatal rats, although saturating concentrations of unlabeled GlySar inhibited the uptake of radiolabeled GlySar by 97%, L-histidine had no effect on GlySar uptake (Fig. 7d). Similar results were obtained when GlySar uptakes were performed in cortical and cerebellar brain slices (data not shown). Thus, the results in rats corroborated our findings in mice, supporting the tenet that while PepT2 might have a greater role in peptide/mimetic uptake in brain parenchyma during early development, PhT1 might be more important in adults.

Discussion

There is a paucity of data on the expression and functional activity of SLC15 family members in different brain regions. Previous immunofluorescence experiments in rats have demonstrated that PepT2 was widely distributed throughout the brain with its strongest expression being observed in epithelial cells of the choroid plexus from both adult and neonatal animals (Shen *et al.* 2004). Moreover, the expression levels of PepT2 protein were highest in foetal cerebral cortex, decreasing monotonically to only 14% of this maximal value in adult rats. This finding was consistent with PepT2 being expressed in the astrocytes and neurons of neonatal rats but only in the neurons of adult rats. However, these studies did not evaluate the activity of PepT2 as a function of age or brain region. Previous autoradiography experiments in mice showed that PepT2 ablation significantly modified the spatial distribution of GlySar and cefadroxil in brain (Smith *et al.* 2011), but this and prior studies (Shen *et al.* 2004) were insufficient in evaluating other (non-PepT2) peptide transporters and the effect of age on regional brain activity.

In the present study, we report several new findings regarding the expression and functional activity of SLC15 family members in regional brain slices from rodents. In
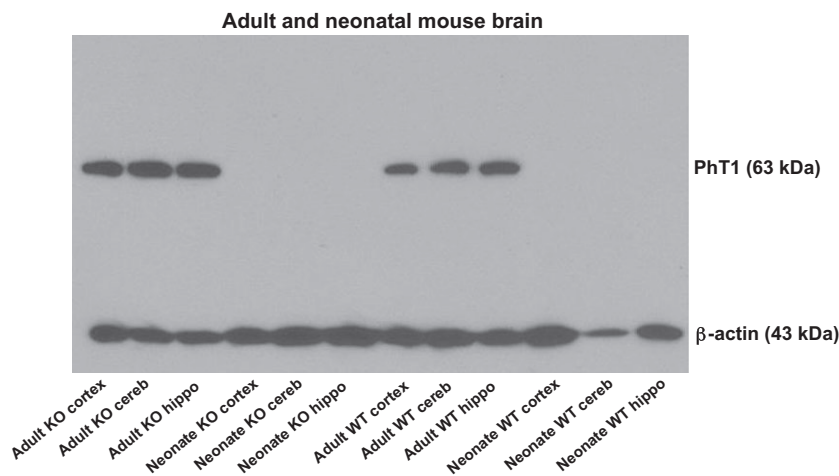
Adult and neonatal mouse brain

PhT1 (63 kDa)

β-actin (43 kDa)

Adult KO cortex, Adult KO cereb, Adult KO hippo, Neonate KO cortex, Neonate KO cereb, Neonate KO hippo, Adult WT cortex, Adult WT cereb, Adult WT hippo, Neonate WT cortex, Neonate WT cereb, Neonate WT hippo

Fig. 2 Immunoblots of PhT1 protein expression in the cerebral cortex, cerebellum (cereb) and hippocampus (hippo) of adult and neonatal wildtype (WT) and PepT2 null (KO) mouse brain. Beta actin served as a loading control.

particular, we found the following: (i) PepT2 and PhT1 transcripts were expressed in the cerebral cortex, cerebellum and hippocampus of mice, whereas PepT1 and PhT2 transcripts had minimal or no expression in these brain regions, (ii) PhT1 protein was present in all three brain regions of adult but not neonatal mice; expression levels increased steadily in rats as a function of advancing age, (iii) the functional activity of PepT2 dominated in neonatal mice and rats but the activity of PhT1 dominated in adult mice and rats, (iv) the cerebral cortex, cerebellum and hippocampus behaved similarly with respect to the uptake of GlySar in these tissues (i.e. no regional differences), (v) the great majority of GlySar uptake was inhibited by L-His (*Max Inh* $\approx$ 80% and $IC_{50} \approx 1$ μM) in adult mice lacking PepT2 and (vi) GlySar uptake, inhibition and SLC15 protein dominance in the developing brain were similar between mice and rats (i.e. no species differences). Taken together, it appears that PepT2 and PhT1 may have reciprocating relevance in brain parenchyma with the former being more important in neonatal rodents and the latter being more important in the adult. The reason why these peptide transporters are regulated in such a manner is unclear.

The physiological, pharmacological and pathological relevance of PhT1 has been difficult to study because definitive information on its cellular and subcellular distribution in brain is lacking. Yamashita *et al.* (1997) reported in adult rats that PhT1 mRNA was widely distributed throughout the brain with intense hybridization signals in hippocampus, choroid plexus, cerebellum and pontine nucleus. The signals were located in both neuronal and non-neuronal cells in these areas. Based on their findings, the authors speculated that PhT1 might be involved in the homeostasis of histamine in brain (i.e. after histidine uptake and decarboxylation), in the termination of neuromodulator effects (i.e. after synaptic and/ or astrocytic uptake of small peptides) and the removal of neuropeptide metabolic fragments from synaptic regions. However, there have been questions raised over whether

PhT1 is expressed on the plasma membrane of mammalian cells (Herrera-Ruiz and Knipp 2003; Daniel and Kottra 2004; Kamal *et al.* 2008). As demonstrated previously by our laboratory in choroid plexus from adult rats (Novotny *et al.* 2000; Teuscher *et al.* 2000, 2001; Shu *et al.* 2002), and by others in synaptosomes from adult rat cerebellum and cerebral cortex (Fujita *et al.* 1999, 2004) and astroglia-rich primary cultures from neonatal rats (Dringen *et al.* 1998; Dieck *et al.* 1999), it is unlikely that PhT1 is active at the plasma membrane of these preparations. This assertion is based on functional studies in which neither an inhibitory effect of excess L-histidine on dipeptide uptake nor an inhibitory effect of excess dipeptide on L-histidine uptake was observed. Instead, immunohistochemical analyses showed that PhT1 co-localized with a late endosome/ lysosome marker in RAW264.7 and COS7 transfected cells (Sasawatari *et al.* 2011) and another study using immuno-fluorescence had PhT1 co-localizing with a marker of early endosomes in HEK293T cells (Lee *et al.* 2009). However, in contrast to that cell line data, the current study on fresh adult brain slices demonstrated that PhT1 had a role in tissue peptide uptake. The impact of L-histidine on GlySar uptake is very unlikely because of an effect on PhT2-mediated transport given the almost complete lack of expression of that transporter in the brain slices.

Our molecular and functional studies in regional brain slices suggest a role for PhT1 in the age-dependent cellular uptake of peptides/mimetics and L-histidine in brain parenchyma, the cell type of which needs further study. The results in mice and rats further demonstrated that PhT1 protein expression was increased developmentally and in a similar manner to that of the histaminergic system (i.e. histidine decarboxylase and the histamine receptors) (Martres *et al.* 1975; Tran *et al.* 1980; Subramanian *et al.* 1981; Reiner *et al.* 1988; Toledo *et al.* 1988). PepT2 protein expression, on the other hand, decreased with age in rats (Shen *et al.* 2004). Thus, PhT1 may have an important role,
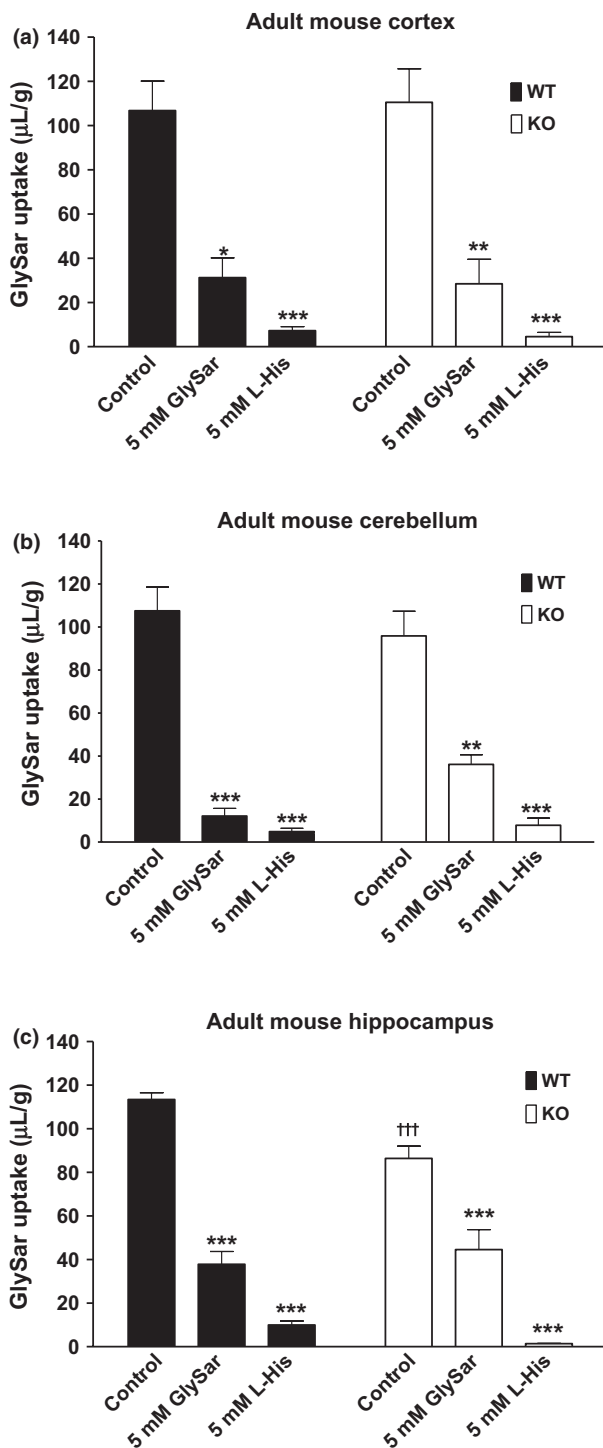
Adult mouse cortex

(a)

Adult mouse cerebellum

(b)

Adult mouse hippocampus

(c)

Fig. 3 Effect of potential inhibitors, glycylsarcosine (GlySar) and histidine (L-His), on the uptake of 2 μM [^{14}C]GlySar in brain slices prepared from the cerebral cortex (a), cerebellum (b) and hippocampus (c) of adult wildtype (WT) and PepT2 null (KO) mouse brain. Values are mean ± SE ($n = 6$). $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$ are compared with control in the same genotype and $^{†††}p \leq 0.001$ is compared with same treatment in the other genotype.

Table 2 Dose-response parameters of the inhibition of GlySar (Uptake) by L-histidine (Inh) in different regions of adult brain from PepT2 null mice

Tissue	No Inh (μL/g)	I_{max} (μL/g)	IC_{50} (μM)	r^2	Max Inh (%)
Cortex	100 ± 5	82 ± 6	0.76 ± 0.27	0.942	82
Cerebellum	85 ± 5	70 ± 6	0.80 ± 0.35	0.949	82
Hippocampus	89 ± 4	69 ± 4	1.25 ± 0.38	0.951	78

Data shown as mean ± SD ($n = 3$ per tissue region). Dose-response parameters were derived from the equation: Uptake = No Inh $- (I_{max} \bullet Inh)/(IC_{50} + Inh)$ where No Inh is the uptake of GlySar in the absence of L-histidine; I_{max} is the maximal inhibition of GlySar uptake by L-histidine; IC_{50} is the concentration of L-histidine at which one-half the I_{max} is reached; and r^2 is the coefficient of determination. Max Inh (%) is calculated as: 100 • I_{max} divided by No Inh, and represents the maximal inhibition observed when the concentration of L-histidine $\gg IC_{50}$ (expressed as percentile).
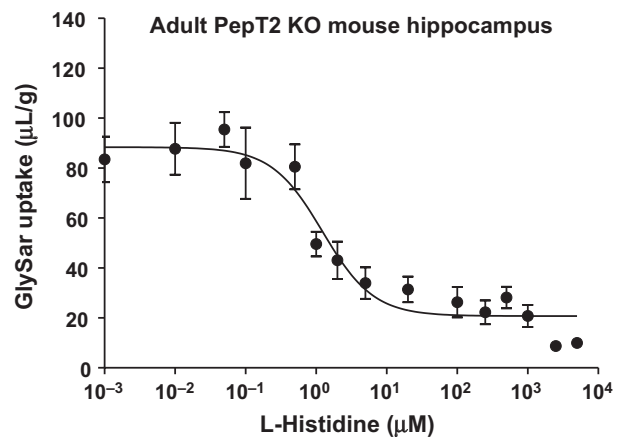

Fig. 4 Dose-response inhibition of 2 μM [^{14}C]glycylsarcosine (GlySar) uptake by L-histidine in brain slices prepared from the hippocampus of adult PepT2 null (KO) mice. Similar results were obtained in the cerebral cortex and cerebellum of mice (data not shown). Values are mean ± SE ($n = 3$).

one that is developmentally regulated, in peptide/mimetic and histidine trafficking in brain. The developmental change in PhT1 protein expression matched the change in L-histidine-inhibitable GlySar uptake in both mouse and rat brain slices offering further evidence that PhT1 (rather than PhT2) was responsible for that transport.

Even though there was a good congruence between developmental changes in PhT1 protein expression and functional activity, brain slices from neonatal mice and rats expressed PhT1 mRNA but not PhT1 protein. The cause of this repression of translation in the neonate and whether it can be manipulated is unknown. It is also unclear as to why a marked residual uptake of radiolabeled GlySar remains in neonatal brain slices (i.e. ≈ 40%) during 5 mM GlySar
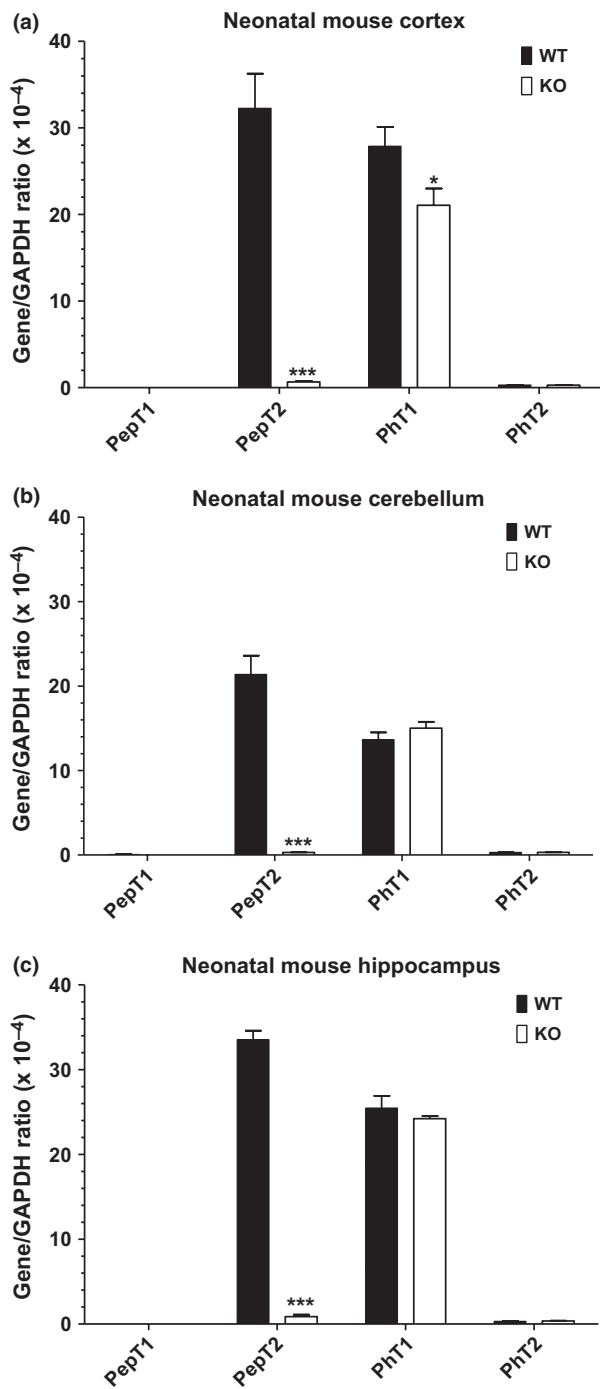
Fig. 5 Real-time PCR of proton-coupled oligopeptide transporter (POT) transcripts in the cerebral cortex (a), cerebellum (b) and hippocampus (c) of neonatal wildtype (WT) and PepT2 null (KO) mouse brain. Data are reported as the ratio of POT to GAPDH determined in each sample. Values are mean ± SE ($n = 3$). *$p \leq 0.05$ and ***$p \leq 0.001$ are compared with WT mice.

Fig. 6 Effect of potential inhibitors, glycylsarcosine (GlySar) and histidine (L-His), on the uptake of 2 μM [^{14}C]GlySar in brain slices prepared from the cerebral cortex (a), cerebellum (b) and hippocampus (c) of neonatal wildtype (WT) and PepT2 null (KO) mouse brain. Values are mean ± SE ($n = 6$). *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are compared with control in the same genotype and $^{†††}p \leq 0.001$ is compared with same treatment in the other genotype.

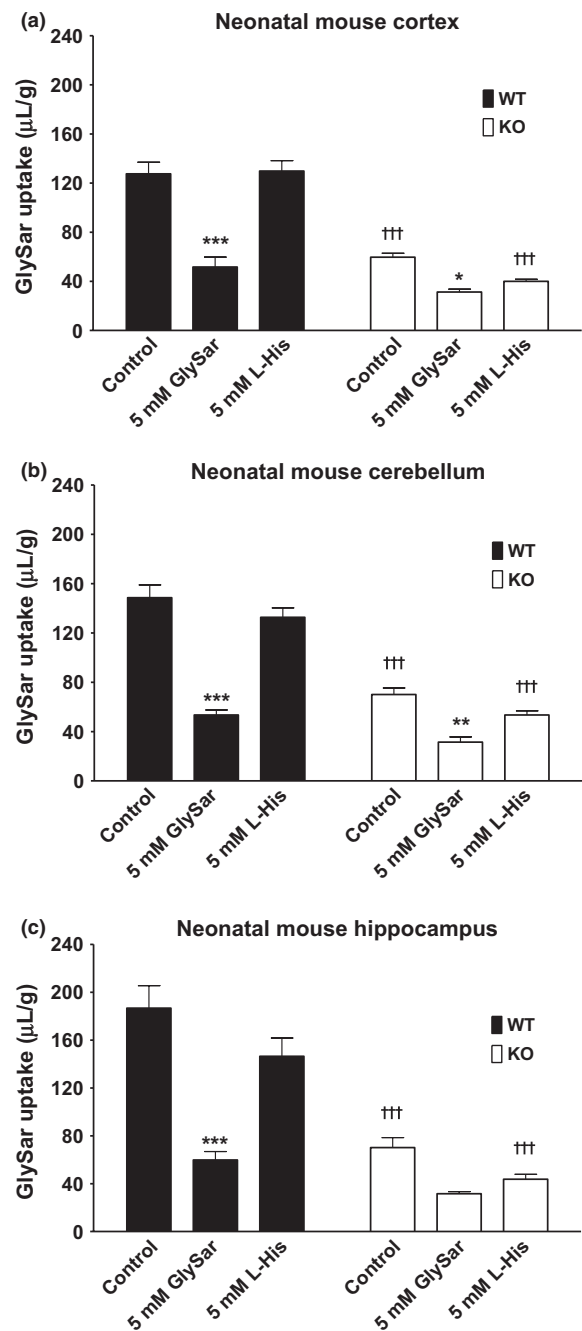inhibition in wildtype mice or in PepT2 null animals (see Fig. 6). Although speculative, this residual activity may reflect a greater passive and/or low-affinity endocytic component when compared with adult mice. Future studies will be needed to address the validity of these proposed mechanisms.

(a) PhT1 mRNA developing rat brain

(b) PhT1 protein developing rat brain

(c) Adult rat hippocampus
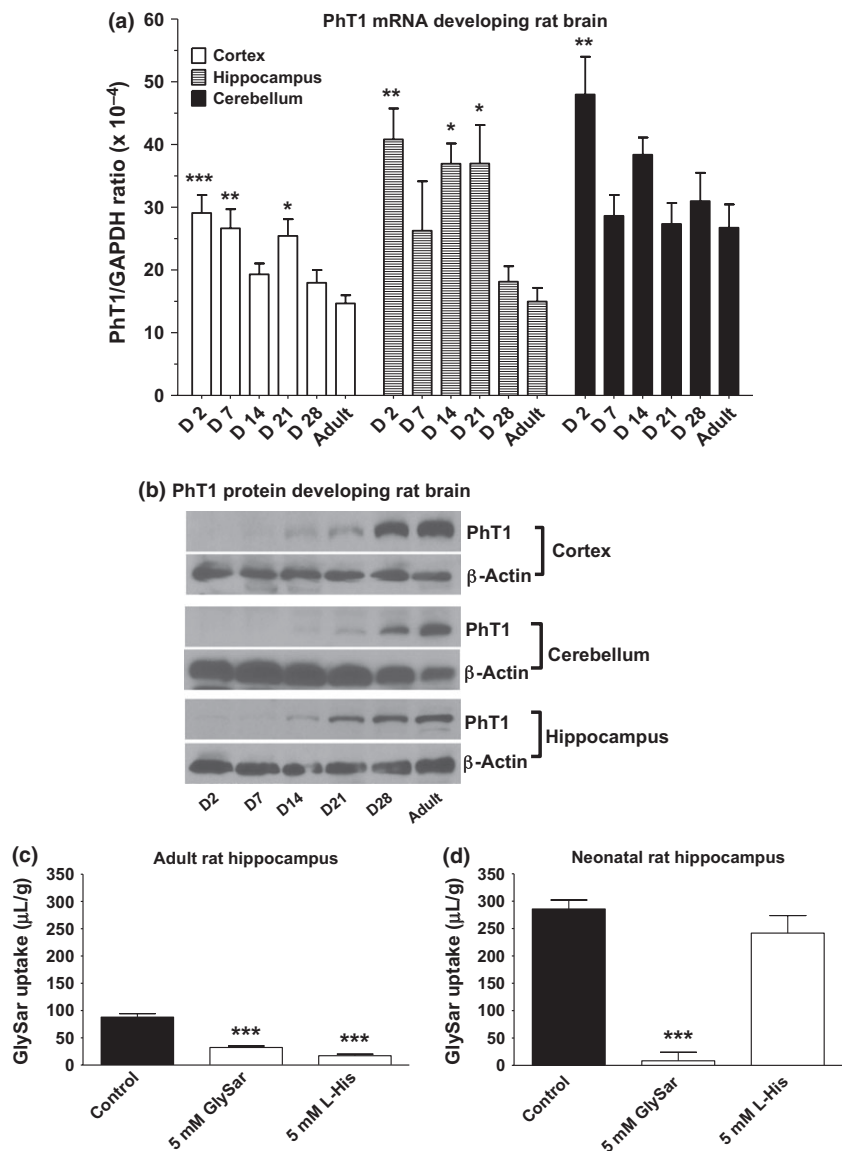
(d) Neonatal rat hippocampus

Fig. 7 Real-time PCR of PhT1 transcripts (a) and immunoblots of PhT1 protein expression (b) in the cerebral cortex, cerebellum and hippocampus of rat brain from day 2 (D2) to week 8 (Adult), and effect of potential inhibitors, glycylsarcosine (GlySar) and histidine (L-His), on the uptake of 2 μM [^{14}C]GlySar in brain slices prepared from the hippocampus of adult (c) and neonatal (d) rat brain. Similar functional results were obtained in the cerebral cortex and cerebellum of rats (data not shown). Values are mean ± SE ($n = 6$). *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are compared with adult (panel a) and control (panels c and d) animals.

Discerning the localization and functional relevance of PhT1 in brain would extend our understanding of neuropeptide trafficking, histidine transport and the potential for drug delivery to neuronal and non-neuronal cell types. Histamine synthesis in the brain is dependent upon the availability of L-histidine and the activity of histidine decarboxylase (Hough and Leurs 2006; Haas *et al.* 2008). As much of the research effort has focused on the enzymatic activation of histamine via histidine decarboxylation, little attention has been paid to the mechanism by which L-histidine gains entry into neurons. Indeed, neuronal entry of histidine is believed to occur by L-amino acid transporters (e.g. SLC38 family) (http://slc.bioparadigms.org). We postulate that PhT1 may also have a major influence in histidine uptake and, therefore, histamine homeostasis. However, the relative importance of various amino acid transporters versus that of PhT1 in transporting L-histidine in brain is unknown. This knowledge is critical because significant changes in brain histamine levels have been observed in several neurological diseases, such as multiple sclerosis, Alzheimer's disease, Downs' syndrome and Wernicke's encephalopathy, making the histaminergic system a prime candidate for novel drug targets. The finding that PhT1-deficient mice had severe decreases in toll-like receptor 9- and nucleotide-binding oligomerization domain-containing protein 1-mediated cytokine production suggests that PhT1 may also have a role in regulating innate immune responses (Sasawatari *et al.* 2011). Finally, several papers have suggested a relationship between PhT1 mutations and systemic lupus erythematosis susceptibility (Han *et al.* 2009; He *et al.* 2010; Lee *et al.* 2013), and one paper proposed PhT1 as a possible target for therapeutic intervention in human lupus (Baccala *et al.* 2013).

In concluding, there are marked developmental changes in the protein expression and functional activity of PhT1 and

PepT2 in brain, with the former predominating in adult and the latter in neonate. These changes may impact the interstitial fluid concentration and biological activity of endogenous peptides and peptide/mimetic drugs.

Acknowledgments and Conflict of interest disclosure

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