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# Peptide transporter 2 (PEPT2) expression in brain protects against 5-aminolevulinic acid neurotoxicity

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**Abstract**

The proton-coupled oligopeptide transporter PEPT2 (or *SLC15A2*) is the major protein involved in the reclamation of peptide-bound amino acids and peptide-like drugs in kidney. PEPT2 is also important in effluxing peptides and peptidomimetics from CSF at the choroid plexus, thereby limiting their exposure in brain. In this study, we report a neuroprotective role for PEPT2 in modulating the toxicity of a heme precursor, 5-aminolevulinic acid (5-ALA). Our findings demonstrate that in PEPT2-deficient mice, 5-ALA administration results in reduced survivability, a worsening of neuromuscular dysfunction, and CSF concentrations of substrate that are 8–30 times higher than that in wild-type control animals. The ability of PEPT2 to limit 5-ALA exposure in CSF suggests that it may also have relevance as a secondary genetic modifier of conditions (such as acute hepatic porphyrias and lead poisoning) in which 5-ALA metabolism is altered and in which 5-ALA toxicity is important.

**Keywords:** 5-aminolevulinic acid, brain, expression, neurotoxicity, peptide transporter 2.

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Peptide transporter 2 (PEPT2) (also known as *SLC15A2*) is a member of the proton-coupled oligopeptide transporter (POT) family that is divided into two subfamilies, including the peptide transporters (i.e. PEPT1 and PEPT2) and the peptide/histidine transporters (i.e. PHT1 and PHT2) (Daniel and Kottra 2004; Smith *et al.* 2004). PEPT2 is considered a high-affinity transporter (i.e. with $K_m$ values in the micromolar range) and is abundantly expressed in the apical membrane of kidney proximal tubule and choroid plexus epithelial cells (Shen *et al.* 1999, 2004), although it is also expressed in lung, mammary gland, and eye (Daniel and Kottra 2004; Smith *et al.* 2004). In kidney, PEPT2 is the major PEPT involved in the reclamation of peptide-bound amino acids and peptide-like drugs (Daniel and Rubio-Aliaga 2003). In choroid plexus, PEPT2 acts as an efflux transporter, thereby limiting the exposure of peptides (Ocheltree *et al.* 2005) and peptide-like drugs (Shen *et al.* 2007) in CSF and brain. PEPT2 is also believed to have a role in regulating neuropeptide homeostasis at the blood–CSF interface (Teuscher *et al.* 2004; Keep and Smith 2006). Based on its cellular localization, PEPT2 should have profound effects on the systemic exposure and tissue distribution of peptides, peptidomimetics, and peptide-like drugs.

In 2003, our laboratory (Shen *et al.* 2003) developed and validated PEPT2-deficient mice by targeted gene disruption in the hope of delineating the physiological, pharmacological, and toxicological roles of this PEPT in the body. Surprisingly, no obvious phenotypic abnormalities were observed in PEPT2 null animals from our colonies (Shen *et al.* 2003) and from that of Rubio-Aliaga *et al.* (2003). Mutant PEPT2 null mice were viable and fertile, grew to normal size and weight, and had similar blood and urine chemistries when compared with wild-type animals. Moreover, PEPT2 null mice did not exhibit an adaptive upregulation in the expression level of related POT genes (such as PEPT1, PHT1, or PHT2), at least in kidney, choroid plexus, and brain (Ocheltree *et al.* 2005). However, PEPT2 had a major impact on the *in vivo* disposition of a model dipeptide, glycylsarcosine (Ocheltree *et al.* 2005), and an antibiotic drug, cefadroxil (Shen *et al.* 2007). Thus, in PEPT2 null mice, both compounds had significantly greater clearances (two- to threefold) as well as CSF/blood ratios (four- to sixfold). These findings suggested that PEPT2 may play a crucial role in influencing the pharmacologic or toxicologic response to POT substrates.

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*Abbreviations used:* 5-ALA, 5-aminolevulinic acid; LT50, time at which 50% of the animals died; PEPT, peptide transporter; PHT, peptide/histidine transporter; POT, proton-coupled oligopeptide transporter.

5-Aminolevulinic acid (5-ALA) is formed in the liver from glycine and succinyl coenzyme A, under control of the rate-limiting enzyme 5-ALA synthase and is a precursor of porphyrins and heme (Anderson *et al.* 2001). Four types of acute hepatic porphyria are the result of deficiencies of specific enzymes in heme biosynthesis (i.e. 5-ALA dehydratase, porphobilinogen deaminase, coproporphyrinogen oxidase, and protoporphyrinogen oxidase), all resulting in elevated levels of 5-ALA and porphyrin precursors (Lindberg *et al.* 1999; Anderson *et al.* 2001; Albers and Fink 2004). Acute porphyrias are associated with neuropsychiatric symptoms including mood disorders, aggressiveness, insomnia, hallucinations, seizures, and convulsions (Anderson *et al.* 2001). The mechanisms underlying neurological dysfunction in these disorders are poorly understood. However, it is believed that the accumulation of 5-ALA, originating in non-neural tissues in hepatic porphyrias, is neurotoxic. This may also be the case in lead poisoning, where inhibition of 5-ALA dehydratase and ferrochelatase during heme biosynthesis results in a significant increase in 5-ALA levels (Klaassen 2006). 5-ALA has been shown to affect the glutamatergic and GABAergic systems, inhibit Na-K-ATPase and adenylate cyclase activities, and cause oxidative stress (Anderson *et al.* 2001; Wang *et al.* 2005; Adhikari *et al.* 2006). There have been questions raised over whether brain (and/or CSF) concentrations of 5-ALA reach high enough concentrations in porphyrias to induce some of these effects (Lindberg *et al.* 1999). Recently, however, Wang *et al.* (2005) found that extracellular 5-ALA concentrations as low as 0.01 μmol/L could affect the sodium channel activity in isolated rat hippocampal CA1 neurons.

5-Aminolevulinic acid is a substrate for the PEPTs with $K_m$ values of 226–260 μmol/L in Xenopus *laevis* oocytes and Pichia pastoris yeast cells expressing cloned PEPT2, and in rat choroid plexus whole tissue where PEPT2 is highly expressed (Döring *et al.* 1998; Novotny *et al.* 2000; Ocheltree *et al.* 2004). As 5-ALA has been implicated in the neuropsychiatric manifestations of acute hepatic porphyrias and lead toxicity, we hypothesized that PEPT2 might act as a secondary genetic modifier in the sensitivity of the brain to its toxicologic effects. If PEPT2 has a significant role in limiting the exposure of 5-ALA in the CSF and brain, then the wild-type mice should be better protected from challenge doses of 5-ALA when compared with PEPT2-deficient animals.

## Materials and methods

### Chemicals

[$^{14}$C]5-ALA (55 mCi/mmol) and [$^{3}$H]dextran (MW 70 000) (450 mCi/g) were purchased from American Radiolabeled Chemicals Inc. (St Louis, MO, USA). Radiochemical purity of both compounds was > 98% as determined by HPLC. Unlabeled 5-ALA was obtained from Sigma-Aldrich (St Louis, MO, USA).

### Animals

Studies were performed in accordance with relevant guidelines and regulations as promulgated by the University Committee on Use and Care of Animals at the University of Michigan, Ann Arbor. Wild-type (PEPT2+/+) and PEPT2 null (PEPT2−/−) mice (> 99% C57BL/6 genetic background) between 8 and 10 weeks of age were used for these studies. Mice were kept in a temperature-controlled environment with a 12 h light, 12 h dark cycle and received a standard diet and water *ad libitum* (Unit for Laboratory Animal Medicine, University of Michigan, Ann Arbor, MI, USA). Gender- and weight-matched mice were used for all experiments.

### Pharmacokinetics and tissue distribution of 5-ALA after intravenous dosing

Radiolabeled 5-ALA was dissolved in normal saline and administered to the mice in aqueous solution (5 μL/g body weight). Following sodium pentobarbital anesthesia (50 mg/kg i.p.), wild-type and PEPT2 null mice received [$^{14}$C]5-ALA (10 nmol/g body weight) through a tail vein injection. Blood samples were collected (~20 μL) via tail nicks at 0.25, 1, 2, 5, 10, 15, 20, 30, 45, and 60 min after the intravenous bolus dose. Heparinized blood samples were centrifuged immediately at 3000 *g* for 3 min to obtain plasma. At 60 min, a CSF sample (~5 μL) was taken from the cisterna magna by puncture of the atlantooccipital membrane with a 28-gauge needle. The mouse was immediately decapitated and tissue samples from choroid plexuses (i.e. lateral and fourth ventricles), cerebral cortex, kidney, liver, eye, lung, heart, spleen, small and large intestines, skeletal muscle, ovary, and testes were collected. Kidney was processed as intact tissue for one kidney and as cortex, outer medulla (outer and inner stripes), and inner medulla for the other kidney. [$^{3}$H]Dextran (1 μCi/mouse) was administered intravenously 5 min prior to harvesting the tissues so that [$^{14}$C]5-ALA tissue concentrations could be corrected for the vascular space. Samples were blotted dry, weighed, and then solubilized with 0.4 mL of 1 mol/L hyamine hydroxide, as described by the manufacturer (ICN, Irvine, CA, USA). Cytoscint (6 mL) liquid scintillation cocktail (ICN) was added to each sample (i.e. digested tissue, CSF, and plasma), and radioactivity was measured by a dual-channel liquid scintillation counter (Beckman LS 3801; Beckman Coulter Inc., Fullerton, CA, USA). Corrected tissue concentrations of 5-ALA ($C_{tiss,\ corr}$, nmol/g wet tissue) were calculated as (Keep *et al.* 1999; Ocheltree *et al.* 2005): $C_{tiss,\ corr} = C_{tiss} - DS \times C_b$, where $C_{tiss}$ is the uncorrected 5-ALA tissue concentration (nmol/g), DS is the dextran space (i.e. blood vascular space) in the tissue (mL/g), and $C_b$ is the 5-ALA blood concentration (nmol/mL). Plasma concentration–time curves were fit to a two-compartment model with a weighting factor of unity using WinNonlin (version 5.0.1; Pharsight Inc., Mountain View, CA, USA) and pharmacokinetic parameters (see Table 1) were determined by standard methods. The quality of fit was determined by evaluating the standard error of parameter estimates, by visual inspection of the residual plots, and by the coefficient of determination ($r^2$). Plasma and tissue concentrations of 5-ALA (expressed in micromolar) were calcu-

 

**Table 1** Pharmacokinetics of 5-ALA plasma concentrations in PEPT2+/+ and PEPT2−/− mice after a 10 nmol/g intravenous bolus dose

| Parameter | PEPT2+/+ | PEPT2−/− |
|---|---|---|
| $V_1$ (mL) | 3.45 (0.04) | 2.89 (0.09) |
| $Vd_{ss}$ (mL) | 10.4 (3.1) | 7.39 (1.30) |
| AUC (μmol × min/L) | 617 (39) | 362 (30)** |
| CL (mL/min) | 0.331 (0.097) | 0.587 (0.067)** |
| $K_{10}$ ($min^{-1}$) | 0.106 (0.015) | 0.229 (0.036)* |
| $t_{1/2}$ (min) | 25.8 (10.2) | 10.5 (2.4)* |
| MRT (min) | 32.8 (12.1) | 12.8 (2.9)* |
| $r^2$ | 0.963 (0.012) | 0.981 (0.009) |

$V_1$ is the volume of the central compartment, $Vd_{ss}$ is the volume of distribution steady state, AUC is the area under the plasma concentration–time curve, CL is the total plasma clearance, $K_{10}$ is the elimination rate constant from the central compartment, $t_{1/2}$ is the terminal half-life, MRT is the mean residence time, and $r^2$ is the coefficient of determination. Data are represented as mean (±SE) ($n = 6$). *$p < 0.05$ and **$p < 0.01$ compared with PEPT2+/+ (wild-type) mice. 5-ALA, 5-aminolevulinic acid; PEPT2, peptide transporter 2.

lated by dividing the sample measurements (dpm/mL) by the specific activity of the radiolabeled compound.

### Toxicological studies after subcutaneous dosing of 5-ALA

Dose-ranging studies were initially performed with unlabeled 5-ALA (i.e. subcutaneous doses escalated daily from 500 to 5000 mg/kg) in wild-type and PEPT2 null mice in an attempt to discriminate between genotypes in their survivability. Based on these pilot studies, 24-h survival experiments were subsequently performed in mice after a single 4000 mg/kg dose of 5-ALA, administered subcutaneously (injection volume of 0.2 mL saline), and the results analyzed using a Kaplan–Meier estimator.

### Neuromuscular studies after subcutaneous dosing of 5-ALA

Serial measurements were made on a rotating rod (3.5 cm diameter, 5 rpm velocity plus 0.1 rpm/s), where altered balance times were used as a measure of neurological dysfunction to unlabeled 5-ALA in mice. In these experiments, balance times were evaluated in wild-type and PEPT2 null mice, 30 min after subcutaneous dosing (injection volume of 0.2 mL saline) of 500 mg/kg 5-ALA given daily for 7 days or 100 mg/kg 5-ALA given daily for 30 days. This study design was intended to probe the modulating effect of PEPT2 on 5-ALA neurotoxicity under chronic conditions and at plasma concentrations that would be more reflective of those levels actually observed in patients with hepatic porphyria.

### Systemic exposure and brain distribution studies after subcutaneous dosing of 5-ALA

Following sodium pentobarbital anesthesia (50 mg/kg i.p.), wild-type and PEPT2 null mice received 100 mg/kg of [$^{14}$C]5-ALA as a subcutaneous dose (injection volume of 0.2 mL saline). Blood samples were obtained (~20 μL) via tail nicks at 5, 10, 30, 60, 90, 120, 180, and 240 min after the dose. Heparinized blood samples were centrifuged immediately at 3000 *g* for 3 min to obtain plasma. In some animals, CSF (~5 μL) and choroid plexus samples were obtained at 30 and 240 min. [$^{14}$C]5-ALA tissue concentrations were corrected for vascular space by administering [$^{3}$H]dextran (0.1 μCi/mouse) intravenously 2 min prior to harvesting the tissues. Samples were then collected, processed, and analyzed, as described before. It should be noted that normal endogenous levels of 5-ALA (e.g. about 0.1 μmol/L in plasma and 0.02 μmol/L in CSF) (Gorchein and Webber 1987) are substantially lower than the concentrations generated by exogenously administered [$^{14}$C]5-ALA.

### Statistics

Data are expressed as mean ± SE. Statistical comparisons between wild-type and PEPT2 null mice were performed by a two sample *t*-test (GraphPad Prism, version 3.0; GraphPad Software Inc., San Diego, CA, USA) in which a probability of $p \leq 0.05$ was considered statistically significant. Statistical differences between multiple treatment groups (in the same genotype) were determined by analysis of variance and pairwise comparisons with the control group were made using Dunnett's test. A two-way ANOVA was performed to simultaneously evaluate the effect of two factors (i.e. time and genotype) on a variable.

## Results

### Pharmacokinetic and tissue distribution studies after intravenous dosing

A preliminary study was performed with intravenous dosing of [$^{14}$C]5-ALA in order to probe whether or not PEPT2 expression would influence the pharmacokinetics and tissue distribution of this substrate. As shown in Fig. 1a, the plasma concentrations of 5-ALA were substantially lower in PEPT2 null mice when compared with wild-type control animals. The reduced systemic exposure (i.e. area under the plasma concentration–time curve) reflects the greater clearance of 5-ALA in animals deficient in renal PEPT2 (Table 1). Volume of distribution terms (i.e. $V_1$ and $Vd_{ss}$) were not significantly different between genotypes, but other measures of a faster elimination of 5-ALA from the body were observed in knockout mice (i.e. $K_{10}$, $t_{1/2}$, and mean residence time; Table 1). Differences are also evident when one compares the tissue distribution of 5-ALA between PEPT2+/+ and PEPT2−/− animals. As observed in Fig. 1b 5-ALA concentrations are significantly lower in the choroid plexus, cerebral cortex, kidney (including renal cortex and outer medulla), eye, and blood of PEPT2 null mice. In contrast to tissue and blood, PEPT2−/− mice had a fivefold greater concentration of 5-ALA in CSF and an eightfold greater CSF/blood concentration ratio. This finding is consistent with the action of PEPT2 in effluxing POT substrates from the CSF into choroid plexus epithelial cells. As a result, PEPT2 may have a significant impact on 5-ALA distribution in brain extracellular fluid and, presumably, on its neurotoxicity. Based on these results, subsequent experiments were performed with 5-ALA after subcutaneous dosing, a route that would facilitate the chronic dosing of substrate (see below).

 

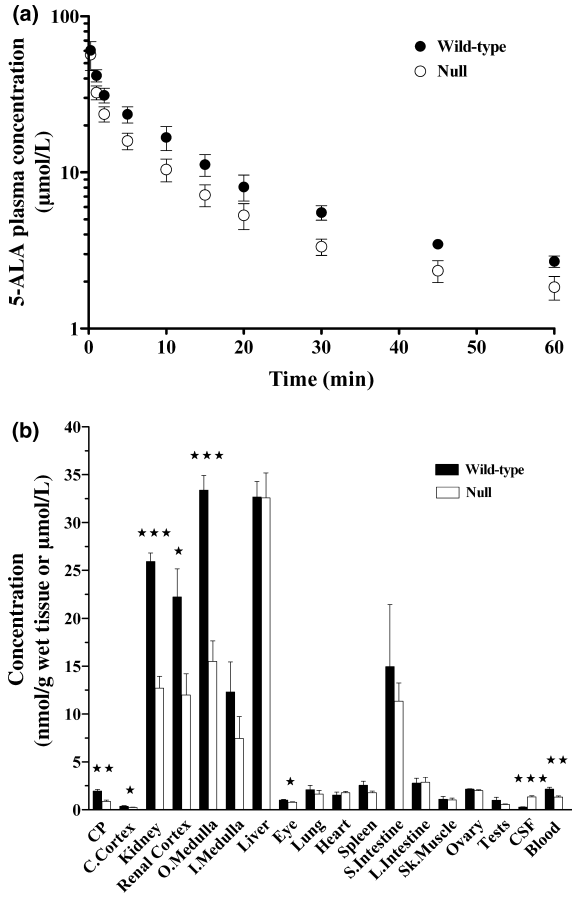


**Fig. 1** Disposition of 5-aminolevulinic acid (5-ALA) in wild-type and peptide transporter 2 null mice following a 10 nmol/g intravenous bolus dose. (a) Plasma concentration-time profiles of 5-ALA. Data are expressed as mean ± SE ($n$ = 6 per genotype). (b) Tissue (nmol/g), CSF (micromolar), and blood (micromolar) concentrations of 5-ALA, 60 min after dosing. Data are expressed as mean ± SE ($n$ = 6 per genotype). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with PEPT2+/+ (wild-type) mice. CP is the choroid plexus, C. Cortex is the cerebral cortex, O. Medulla is the outer medulla, I. Medulla is the inner medulla, S. Intestine is the small intestine, L. Intestine is the large intestine, and Sk. Muscle is the skeletal muscle.

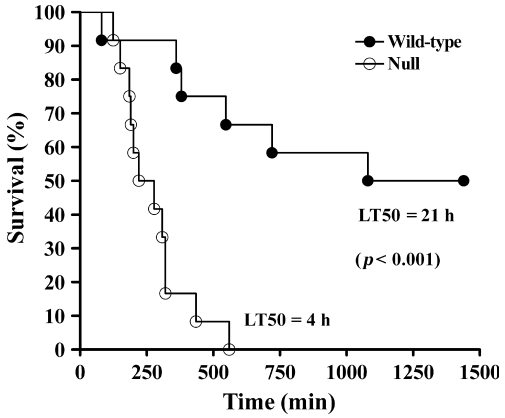


**Fig. 2** Kaplan–Meier survival curves in wild-type and peptide transporter 2 null mice following a single 4000 mg/kg subcutaneous dose of 5-aminolevulinc acid. LT50 is the time at which one-half the animals die ($n$ = 12 per genotype).

## Toxicological studies after subcutaneous dosing

Figure 2 demonstrates that PEPT2 had a major impact on the ability of PEPT2 null mice to survive the toxic insult of a high single dose of 5-ALA (4000 mg/kg). While the time at which 50% of the animals died (i.e. LT50) was 21 h in PEPT2-competent mice, the LT50 for animals lacking the PEPT2 gene was only 4 h ($p < 0.001$). This finding provides strong evidence that PEPT2 confers a neuroprotective advantage in those mice containing PEPT2 protein in brain.

## Neuromuscular studies after subcutaneous dosing

Further evidence for a neuroprotective role of PEPT2 is demonstrated in Fig. 3. Under chronic dosing conditions of 500 mg/kg 5-ALA given daily for 7 days, PEPT2+/+ mice showed no sign of any altered ability to maintain balance on a rotating beam (i.e. rotary rod test) (Fig. 3a). However, the rotary rod times of PEPT2-deficient mice were progressively reduced in response to 5-ALA administration. In particular, neuromuscular dysfunction was observed after 4 days of 5-ALA dosing, and balance times were reduced to 58% of control values at 7 days. This finding was even more convincing when chronic dosing conditions of 100 mg/kg 5-ALA given daily for 30 days were examined (Fig. 3b). In that study, by 30 days, PEPT2+/+ mice had balance times that were 91% of control values while PEPT2−/− mice had balance times that were only 60% of control values (i.e. at day 0). Moreover, there were highly significant correlations as a function of time and genotype ($p < 0.001$ for both factors).

## Systemic exposure and brain distribution studies after subcutaneous dosing

As shown in Fig. 4, there is little difference in the systemic exposure of 5-ALA (100 mg/kg) in wild-type and PEPT2 null mice after subcutaneous dosing. Peak plasma concentrations occurred at 30 min and almost all the 5-ALA was eliminated from the body at 240 min. As a result, accumulation of 5-ALA is not expected when dosed once a day over 7 days or even 30 days. Although the plasma concentrations were similar between PEPT2+/+ and PEPT2−/− mice, CSF concentrations of 5-ALA were eight- and 30-fold greater at 30 min ($p < 0.001$) and 240 min ($p < 0.001$), respectively, in PEPT2 null mice (Fig. 5a). These differences reflected the loss of PEPT2 in apical membranes of choroid plexus epithelial cells and, as a result, 5-ALA concentrations in the choroid plexus whole tissue of PEPT2−/− mice were 8% and 17% of the values in PEPT2+/+ animals at 30 min ($p < 0.001$) and 240 min ($p < 0.01$), respectively (Fig. 5b).

 

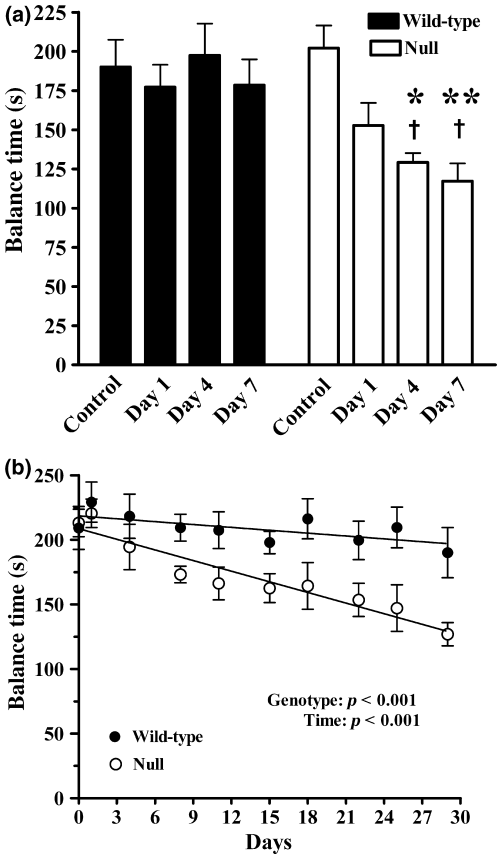


**Fig. 3** Rotary rod test in wild-type and peptide transporter 2 (PEPT2) null mice. (a) 5-Aminolevulic acid was administered daily at 500 mg/kg subcutaneous doses for 7 days, and the balance times were measured pre-dose and 30 min after dosing on days 1, 4, and 7. Data are expressed as mean ± SE ($n$ = 4–8 per genotype). *$p < 0.05$, and **$p < 0.01$ compared with the pre-dose control group of PEPT2 null mice. †$p < 0.05$ compared with PEPT2+/+ (wild-type) mice on the same day. (b) 5-aminolevulinic acid was administered daily at 100 mg/kg subcutaneous doses for 30 days, and the effect was measured pre-dose and 30 min after dosing on days 1, 4, 8, 11, 15, 18, 22, 25, and 29. Data are expressed as mean ± SE ($n$ = 8 per genotype).

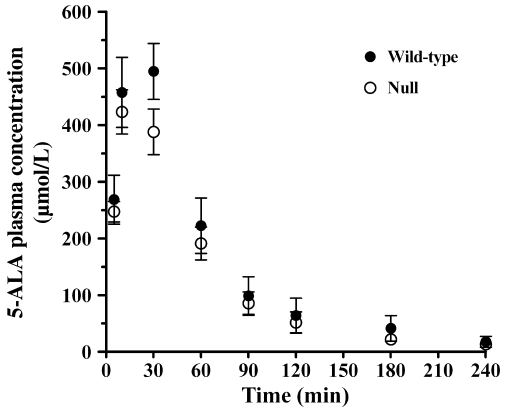


**Fig. 4** Plasma concentration–time profiles of 5-aminolevulinic acid (5-ALA) in wild-type and peptide transporter 2 null mice following a 100 mg/kg subcutaneous dose. Data are expressed as mean ± SE ($n$ = 5 per genotype).

The differences in CSF and choroid plexus concentrations of 5-ALA were not the result of differences in plasma concentration as little or no changes were observed (i.e. less than 25%; Fig. 5c).

## Discussion

Our findings are novel in establishing, for the first time, a neuroprotective phenotype for the POT family member PEPT2. Specifically, we demonstrate: (i) that survivability is reduced in PEPT2-deficient mice after high-dose acute administration of 5-ALA, (ii) that neuromuscular function is worsened in PEPT2-deficient mice after chronic dosing of 5-ALA, and (iii) that the pharmacokinetic profile, as best reflected by the 8–30 times higher CSF levels of 5-ALA in PEPT2−/− mice, is consistent with the pharmacodynamic observations. Moreover, the results are clinically relevant as the 5-ALA plasma levels in this study (i.e. about 23 μmol/L; calculated as area under the plasma concentration–time curve/24 h) are similar to the plasma concentrations of 5-ALA observed in patients during acute attacks of porphyria (i.e. 2–13 μmol/L) (Lindberg *et al.* 1999), supporting the hypothesis that 5-ALA is the neurotoxic agent in porphyrias. Of all the tissues examined, CSF was the only compartment that had higher concentrations of 5-ALA in PEPT2-deficient mice when compared with wild-type animals. This suggests that the greater toxicity of 5-ALA in PEPT2−/− mice is because of changes in CSF (and presumably brain extracellular fluid) concentrations.

Accumulation of the porphyrin precursors, 5-ALA and/or porphobilinogen, have been implicated as causes of the neuropsychiatric symptoms that occur in acute hepatic porphyrias and lead toxicity (Lindberg *et al.* 1999; Anderson *et al.* 2001; Albers and Fink 2004; Klaassen 2006). A number of mechanisms (which may be interrelated) have been proposed by which 5-ALA may affect CNS function. These include effects on the glutamatergic and GABAergic systems, inhibition of Na-K-ATPase and adenylate cyclase activities, and oxidative stress (Anderson *et al.* 2001; Wang *et al.* 2005; Adhikari *et al.* 2006). However, many, but not all (Wang *et al.* 2005), studies have used very high concentrations of 5-ALA (often in the millimolar range) that may not be relevant to porphyrias and Edwards *et al.* (1984) found that acute administration of 5-ALA by subcutaneous or intraperitoneal injection does not significantly affect nociception, CNS excitability, motor coordination, grip, and cardiovascular function in rodents. In the latter study, the authors noted that their results were not consistent with the evidence obtained from *in vitro* studies which suggested that

 

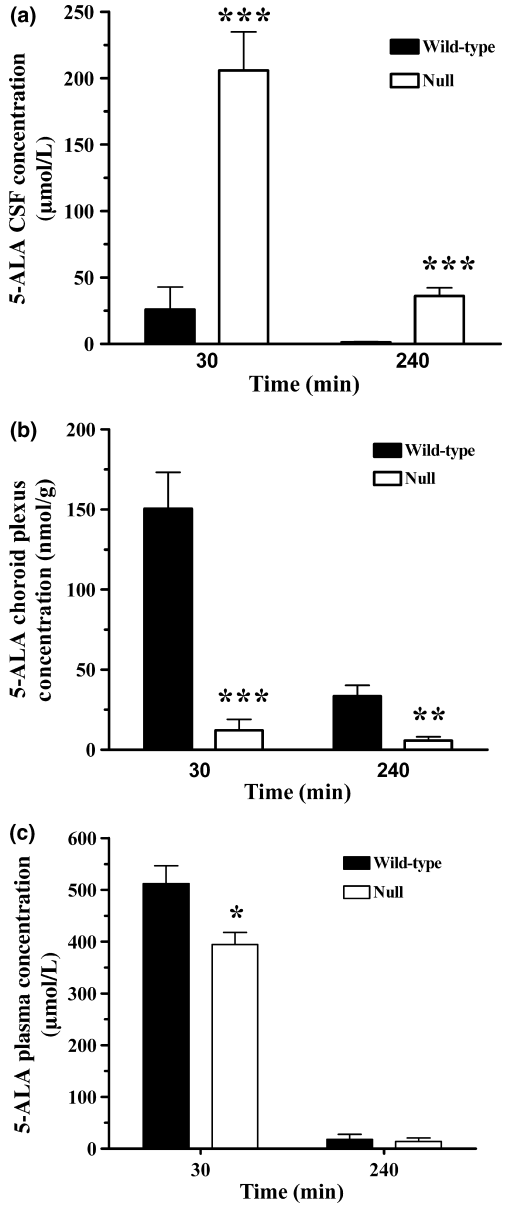


**Fig. 5** Biological concentrations of 5-aminolevulinic acid (5-ALA) in wild-type and peptide transporter 2 (PEPT2) null mice, 30 and 240 min after a 100 mg/kg subcutaneous dose. (a) 5-ALA concentrations in CSF. Data are expressed as mean ± SE ($n = 5$ per genotype). ***$p < 0.001$ compared with PEPT2+/+ (wild-type) mice at the same sampling time. (b) 5-ALA concentrations in choroid plexus. Data are expressed as mean ± SE ($n = 5$ per genotype). **$p < 0.01$ and ***$p < 0.001$ compared with PEPT2+/+ (wild-type) mice at the same sampling time. (c) 5-ALA concentrations in plasma. Data are expressed as mean ± SE ($n = 10$ per genotype). *$p < 0.05$ compared with PEPT2+/+ (wild-type) mice at the same sampling time.

5-ALA has significant neuropharmacological activity. They suggested a need for chronic studies to better understand the relationship between 5-ALA doses and neurotoxicity. Adhikari *et al.* (2006) recently found that chronic administration of low doses of 5-ALA (40 mg/kg in rat) reduced GABAergic receptor density in the brain although they did not report any behavioral data. In our current study, we found that chronic administration of a low dose of 5-ALA (100 mg/kg) for 30 days, which produced pathophysiologically relevant 5-ALA concentrations, could induce behavioral deficits in PEPT2 null mice where the lack of the transporter lead to increased CSF concentrations. It is unlikely that these effects were influenced by a compensatory response to PEPT2 gene disruption as previous studies have indicated that the null mice do not exhibit altered expression levels of related POT genes (Rubio-Aliaga *et al.* 2003; Ocheltree *et al.* 2005; Frey *et al.* 2007).

Targeted gene replacement has allowed scientists to learn more about the physiological, pharmacological, and pathological role of a particular gene product in the body (Capecchi 1994; Elmquist and Miller 2001). However, disruption of a specific gene does not always result in phenotypical abnormalities, especially with respect to knocking out transporter proteins. For example, it was by pure serendipity that mice lacking the multidrug resistance gene 1a were found to be extremely sensitive to the anthelmintic drug, ivermectin (Schinkel *et al.* 1994). During the typical spraying of animals to treat mite infestation, a number of mice died with paralytic symptoms. Apparently, loss of the gene product, p-glycoprotein, at the blood–brain barrier allowed this normally safe pesticide to reach levels in the brain that were 90 times greater than that observed in wild-type mice. Another example involves the breast cancer resistance protein 1 in which null mice did not demonstrate phenotypic aberrations under standard housing conditions (Jonker *et al.* 2002). However, a few mice housed on the top shelf, closest to the fluorescent light source and being fed one particular batch of food containing alfalfa leaf concentrate, developed severe necrotic ear lesions. In this case, high levels of pheophorbide a, a phototoxic porphyrin catabolite of chlorophyll, was formed during the storage of alfalfa. Both examples point to the fact that, on occasion, a phenotypic abnormality is not observed until the mice are challenged in a certain manner. With respect to our PEPT2 knockout mice, chronic dosing of 5-ALA (or a very high single dose) was necessary in order to observe a toxicologic phenotype. This result suggests that human subjects with a reduced functional activity or expression of PEPT2 in brain may be more sensitive to the neurotoxic effects of POT substrates such as 5-ALA.

It should be noted that, as PEPT2-mediated transport is hydrogen ion dependent, functional activity may also change in disease states where CSF pH is altered. For example, respiratory (unlike metabolic) acidosis/alkalosis can cause marked changes in CSF pH (Kazemi and Johnson 1986), and CSF pH levels as low as 6.59 have been recorded in patients with traumatic injury as a result of cerebral ischemia and

increases in CSF lactate, pyruvate, and $pCO_2$ (Shiogai *et al.* 1999). Normal pH in the CSF is 7.3 (Smith *et al.* 2004).

Numerous single nucleotide polymorphisms have been reported for the *SCL15A2* gene in humans (Pinsonneault *et al.* 2004; Terada *et al.* 2004). In the first study (Terada *et al.* 2004), transport activity of genetic variant Arg57His was completely abolished when transiently expressed in both human embryonic kidney 293 cells and *Xenopus laevis* oocytes. The functional impairment by this rare non-synonymous polymorphism (i.e. hPEPT2 R57H) occurred in spite of a conserved protein expression at the cell plasma membrane. In the second study (Pinsonneault *et al.* 2004), a haplotype analysis revealed two main hPEPT2 variants (i.e. hPEPT2*1 and *2), with several phased amino acid substitutions, that were present in substantial frequencies in all ethnic groups tested. When expressed in Chinese Hamster Ovary cells, the $K_m$ values of glycylsarcosine differed significantly (83 μmol/L vs. 233 μmol/L for hPEPT2*1 vs. *2, respectively) as did their sensitivity to proton/substrate symport. These results indicate that polymorphisms in the gene encoding hPEPT2 can alter peptide/mimetic transport, be it from exogenous or endogenous sources. Whether or not genetic polymorphisms of PEPT2 are present in patients with acute hepatic porphyrias or lead poisoning is currently unknown. However, there are a variety of mutations that markedly reduce the activity of specific enzymes in heme biosynthesis (Anderson *et al.* 2001) and, in this context, it is possible that PEPT2 may act as a secondary genetic modifier in making these patients more sensitive to the pharmacologic or toxicologic effects of POT substrates such as 5-ALA.

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