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Histochemical Localization of Palmitoyl Protein Thioesterase-1 Activity

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Abstract

Infantile neuronal ceroid lipofuscinosis (INCL, Infantile Batten disease) is an invariably fatal neurodegenerative pediatric disorder caused by an inherited mutation in the *PPT1* gene. Patients with INCL lack the lysosomal enzyme palmitoyl protein thioesterase-1 (PPT1, EC 3.1.2.22), resulting in intracellular accumulation of autofluorescent storage material and subsequent neuropathology. The *Ppt1*^{-/-} mouse is deficient in PPT1 activity and represents a useful animal model of INCL that recapitulates most of the clinical and pathological aspects of the disease. Preclinical therapeutic experiments performed in the INCL mouse include CNS-directed gene therapy and recombinant enzyme replacement therapy; both seek to re-establish therapeutic levels of the deficient enzyme. We present a novel method for the histochemical localization of PPT1 activity in the *Ppt1*^{-/-} mouse. By utilizing the substrate CUS-9235, tissues known to be positive for PPT1 activity turn varying intensities of blue. Presented here are histochemistry data showing the staining pattern in *Ppt1*^{-/-}, wild type, and *Ppt1*^{-/-} mice treated with enzyme replacement therapy or AAV2/9-PPT1-mediated gene therapy. Results are paired with quantitative biochemistry data that confirm the ability of CUS-9235 to detect and localize PPT1 activity. This new method complements the current tools for the study of INCL and evaluation of effective therapies.

Keywords

Batten disease; lysosomal storage disorder; histochemistry; palmitoyl protein thioesterase-1; enzyme replacement therapy

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1. Introduction

Infantile neuronal ceroid lipofuscinosis (INCL, Infantile Batten disease, Cln1 disease) is a profoundly neurodegenerative lysosomal storage disorder (LSD) that affects children in early infancy. It is caused by mutations in the *PPT1* gene which encodes the lysosomal enzyme palmitoyl protein thioesterase-1 (PPT1). This enzyme is primarily responsible for catalyzing the cleavage of thioester bonds that attach long-chain fatty acids to specific cysteine residues in polypeptides [1]. When the *PPT1* gene is mutated, leading to a deficiency or complete lack of PPT1 activity, intracellular autofluorescent storage material accumulates in the central nervous system (CNS) and to varying degrees in many peripheral organs that express the mannose-6-phosphate receptor including kidney, liver, and heart. Concurrent with this accumulation are astrogliosis, cortical atrophy, microglial activation, apoptosis, and loss of gamma-aminobutyric acid (GABA) neurons [2]. Children with this disorder experience a rapid progression of symptoms, beginning at 6–12 months of age, that includes visual loss, motor impairments, seizures, and premature death as early as 6 years of age [3–7].

A useful animal model of INCL is found in the *Ppt1*-knockout mouse (*Ppt1*^{−/−}). This animal is PPT1-deficient and recapitulates many key aspects of the human disease. *Ppt1*^{−/−} mice experience visual deficits, motor abnormalities, myoclonic seizures, and premature death [8]. There is currently no known cure for INCL. However, pre-clinical experiments in the PPT1-deficient mouse suggest that some treatments may delay or lessen the severity of symptoms. All of these treatments address, either directly or indirectly, the central issue involved in INCL: lack of PPT1 enzyme activity. Until recently it has been impossible to detect PPT1 activity *in situ*. Polyclonal antibodies exist that are useful in localizing PPT1 protein; however, immunolocalization techniques may not correlate with enzyme activity. Localizing PPT1 activity *in situ* would greatly enhance our ability to develop and analyze novel therapies designed to replace PPT1. Here we describe the development and application of a histochemical stain (CUS-9235) for detecting PPT1 activity.

2. Methods

2.1 Ethics statement

All animal procedures were approved by the Institutional Animal Studies Committee at Washington University School of Medicine and were in accordance with the guidelines of the National Institutes of Health.

2.2 Animals

The *Ppt1*^{−/−} mouse was created by eliminating the last exon of the *PPT1* gene using a targeted disruption strategy [8]. This mutation was backcrossed to the C57BL/6 mouse for more than 10 generations, ultimately establishing colonies of *Ppt1*^{−/−} and *Ppt1*^{+/+} (WT) homozygotes that were maintained by MSS. Males and females of each genotype were used at various ages and under different treatment conditions for histochemical experiments. All animals were housed in an animal facility at Washington University School of Medicine in St. Louis, MO. Colonies were maintained under a 12 h light/dark cycle and had *ad libitum* access to food and water.

2.3 Enzyme therapy

Recombinant human PPT1 was expressed and purified as previously described [9]. Enzyme was stored at -80°C in aliquots at a concentration of 1.5 mg/mL and thawed only as needed. Adult (approximately 2 months of age) *Ppt1*^{-/-} mice (n = 1 per dose) were injected with 250 μL of enzyme (corresponding to approximately 15 mg/kg) or 25 μL of enzyme (1.5 mg/kg) via tail vein. Neonatal *Ppt1*^{-/-} mice (n = 4 per group) were injected on post-natal day (PND) 1 or 2 with 100 μL of enzyme via the superficial temporal vein. Mice were euthanized 2 h following enzyme injection. WT and *Ppt1*^{-/-} mice were age-matched.

2.4 Gene therapy

The rAAV2/9-PPT1 vector used for these studies was produced as previously described [10, 11] with the key difference being that we used the AAV9 capsid protein instead of AAV5. The AAV9 capsid is reported to have widespread distribution throughout the brain following intracranial injection, primarily transduces neurons, and may be capable of crossing the blood-brain barrier (BBB) [12]. *Ppt1*^{-/-} mice (n = 2) received systemic gene therapy (1×10^{11} vg/mouse) via tail vein injection at 28 days of age. Four weeks following injection, mice were euthanized via anesthesia overdose (Fatal-Plus Solution, Vortech Pharmaceuticals, Dearborn, MI, USA).

2.5 Tissue processing and histochemistry

Following euthanasia, mice were dissected to acquire brain, kidney, liver, spleen, and heart for both histochemical and quantitative biochemical analyses. Unfixed tissues (whole kidney, one brain hemisphere, and samples of liver, spleen, and heart) were placed directly into Optimal Cutting Temperature Compound (OCT; Sakura Finetek USA, Inc., Torrance, CA), frozen on dry ice, and stored at -80°C prior to sectioning. In those cases where whole infant bodies were used, euthanized mice were submerged directly into OCT and frozen on dry ice. Sixteen micrometer cryosections were mounted directly onto *Superfrost Plus* slides (Thermo Fisher Scientific, Waltham, MA, USA). A water repellent barrier was created around the mounted tissue using the Super HT PAP pen (Biotium, Inc., Hayward, CA, USA) and tissue was fixed with 4% paraformaldehyde in PBS for 20 minutes at 4°C . Following fixation the slides were rinsed extensively with 0.2 M sodium acetate buffer (pH 4.5). After rinsing, each slide was covered with approximately 1 mL solution which consisted of: 250 μL 0.2 M sodium acetate, 628.5 μL sterile H_2O , 10 μL 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA), 40 μL β -cyclodextrin (50 mg/mL), 25 μL 0.2 M ferrocyanide, 25 μL 0.2 M ferricyanide, 20 μL β -glucosidase (50 mg/mL) [13], 6.47 μL 1 M dithiothreitol, 20 μL dimethyl sulfoxide (DMSO), and 20 μL CUS-9235 (50 mg/mL in DMSO). This substrate (Figure 1) was custom synthesized by Matreya, LLC (State College, PA, USA) and is structurally similar to the fluorogenic PPT1 substrate previously described [13] with substitution of the 5-bromo-4-chloro-3-indole for the 4-methylumbelliferone (MU). Components of the CUS-9235 staining solution are best added in the order listed above so as to minimize precipitation. Slides were incubated in a humidified chamber overnight at 37°C . Approximately 16 h later slides were rinsed with copious amounts of H_2O and counterstained for 2 min with Nuclear Fast Red (Sigma-Aldrich, St. Louis, MO, USA). After a final rinse with H_2O , the slides were left at room temperature to dry overnight, then

covered using microscope cover glass (Thermo Fisher Scientific, Waltham, MA, USA) and Cytoseal (Electron Microscopy Sciences, Hatfield, PA, USA).

2.6 Biochemical assays

Samples of the same organs used for staining (whole kidney, one brain hemisphere, liver, spleen, and heart) were flash frozen in liquid nitrogen and homogenized in buffer as previously described [13]. Following centrifugation the supernatant was incubated with the fluorogenic substrate 4-methylumbelliferyl-6-thiopalmityl- β -glucoside in a 37°C water bath for 1 h. The reaction was stopped with 500 μ L of a 0.1 M sodium carbonate/sodium bicarbonate buffer. Fluorescence emission was measured at 448 nm following excitation at 365 nm in a Hitachi F-2000 fluorescence spectrophotometer (Hitachi, Pleasanton, CA). Measurements were compared to a standard curve ranging from 0.5 to 5 mM of 4-methylumbelliferone (4-MU). PPT1 activity was normalized to total protein measured using a Coomassie dye-binding assay (Bio-Rad Laboratories, Hercules, CA) and data are expressed as nmol of substrate cleaved per mg of protein per h (nmol/mg/h).

3. Results

3.1 PPT1 distribution in normal and enzyme-treated newborn mice

Entire newborn animals were stained for PPT1 activity (Figure 2). PPT1 activity is indicated by a blue color and the tissue was counterstained with nuclear fast red. *Ppt1*^{-/-} mice (Fig. 2a) show no PPT1 activity in any organ, with red being the only color present across the entire body. In contrast, WT mice (Fig. 2b) exhibit a number of areas of intense blue staining. PPT1 activity is apparent in the brain, eye, bone tissue, thymus, gut, and kidney. There appears to be relatively little PPT1 present in the skin, brown fat, lung, and heart. *Ppt1*^{-/-} mice treated with intravenous recombinant enzyme (Fig. 2c) have a different distribution of PPT1 activity. Intense blue staining is observed in enzyme-injected animals in liver, spleen, kidney, heart, eye, brown fat, skin, lung, thymus, bone marrow, and gut. However, there is relatively little PPT1 staining in the brain compared to peripheral tissues.

3.2 Quantitative enzyme assay in newborn tissues

We performed PPT1 assays to determine the level of enzyme activity in tissues collected from neonatal WT, *Ppt1*^{-/-} mice, and *Ppt1*^{-/-} mice treated with intravenous enzyme (Figure 3). While enzyme activity is nearly undetectable in the *Ppt1*^{-/-} infant brain, WT infant brains contain approximately 70.7 nmol/mg/hr. Intravenous enzyme treatment increases PPT1 activity in the *Ppt1*^{-/-} infant brain to approximately 32.6 nmol/mg/hr. Treatment with enzyme increases PPT1 activity in the *Ppt1*^{-/-} infant heart to 529.2 nmol/mg/hr, a five-fold increase over WT levels (88.2 nmol/mg/hr), while untreated *Ppt1*^{-/-} heart tissue contain only 3.2 nmol/mg/hr. Enzyme activity in the livers of *Ppt1*^{-/-} mice is very low (5.8 nmol/mg/hr), while treatment with enzyme increases this number to 459.6 nmol/mg/hr. This is a four-fold increase in the level of PPT1 activity compared to infant WT liver tissue (88.1 nmol/mg/hr). Kidney tissue from enzyme-treated *Ppt1*^{-/-} mice contains 903.2 nmol/mg/hr PPT1 activity, nearly 10-times the amount in WT kidney (96.6 nmol/mg/hr) and over 50-times that seen in untreated *Ppt1*^{-/-} kidney (15.7 nmol/mg/hr). Finally, intravenous enzyme treatment increases PPT1 activity in the spleen from 5.6

nmol/mg/hr (untreated *Ppt1*^{-/-} infants) to 305.2 nmol/mg/hr in treated *Ppt1*^{-/-} infants, while infant WT spleen contains 57.0 nmol/mg/hr. Of note, PPT1 activity in the *Ppt1*^{-/-} brain was increased to 45% of WT levels 2 h after enzyme injection, whereas activity levels in peripheral tissues were much higher than WT, ranging from four-fold (liver) to over 10-fold (kidney) over normal tissues.

3.3 PPT1 distribution in normal and enzyme-treated adult mice

Ppt1^{-/-} mice (Figure 4a) exhibit no apparent PPT1 staining in the kidney. WT mice (Fig. 4b) show staining throughout the renal cortex as well as the renal medulla, with the most intense stain present in the cortex among the glomeruli and associated tubules. In *Ppt1*^{-/-} mice receiving 25 μ L enzyme (1.5 mg/kg; Fig. 4c), some PPT1 activity is detected in the renal cortex but not the medulla; this staining clearly does not reach WT levels. In contrast, the kidney from *Ppt1*^{-/-} mice receiving 250 μ L of enzyme (15 mg/kg; Fig. 4d) exhibit intense blue staining in the renal cortex, clearly more intense than the stain in WT mice. However, even in those mice receiving the larger dose of enzyme, relatively little PPT1 activity is detected in the renal medulla.

Staining in heart presents a somewhat similar pattern to the kidney. There appears to be no PPT1 activity in *Ppt1*^{-/-} heart (Fig. 4e) and moderate activity in WT heart (Fig. 4f). The lower dose of enzyme therapy (1.5 mg/kg) did not result in detectable staining in the *Ppt1*^{-/-} heart (Fig. 4g), and the full 250 μ L dose (15 mg/kg; Fig. 4h) supplies PPT1 to the heart at a level similar to that seen in WT heart.

A similar picture emerges when examining sections of liver. There are no traces of blue stain to be found in the liver of *Ppt1*^{-/-} mice (Fig. 4i), while PPT1 appears to be widely distributed throughout the liver of WT mice (Fig. 4j). Enzyme therapy clearly restored some PPT1 activity in the livers of *Ppt1*^{-/-} mice with considerably more intense staining in mice receiving 250 μ L (Fig. 4l) compared to 25 μ L (Fig. 4k). An interesting difference in the distribution of PPT1 activity is observed between WT mice (Fig. 4n) and *Ppt1*^{-/-} mice receiving 250 μ L of enzyme (Fig. 4p) when viewed at higher magnification. Staining in WT liver appears to be spread throughout the tissue in a punctate pattern, indicating high levels of PPT1 activity in parenchymal cells (likely to be Kupffer cells) as well as some sinus lining cells. This is in distinct contrast to the pattern of staining seen in enzyme-treated *Ppt1*^{-/-} liver tissue which appears to be a uniform blue with the absence of a punctate pattern. In addition, sinus-lining cells in enzyme-treated *Ppt1*^{-/-} liver appear largely devoid of PPT1 activity.

3.4 PPT1 distribution in AAV2/9-PPT1-treated mice

Compared to the same tissue from *Ppt1*^{-/-} (Figure 5a) and WT mice (Fig. 5b), kidney tissue from *Ppt1*^{-/-} mice injected intravenously 4 weeks prior with AAV2/9-PPT1 (Fig. 5c) stained a uniform, intense blue across the entire organ. In contrast to enzyme-treated mice, there appears to be little or no distinction in PPT1 activity between the renal cortex and renal medulla. PPT1 staining is considerably more robust in AAV-treated mice compared to untreated *Ppt1*^{-/-} mice or WT mice. Histochemical staining with CUS-9235 reveals high levels of PPT1 activity in heart tissue from mice treated with AAV2/9 (Fig. 5f). Distribution

appears to be uniform across the heart and more intense than WT (Fig. 5e) or *Ppt1*^{-/-} tissue (Fig. 5d). This pattern is similar in liver tissue. Staining in *Ppt1*^{-/-} mice treated with AAV2/9-PPT1 (Fig. 5i) is intense and uniform regardless of anatomy. There appears to be a distinct increase in liver PPT1 activity with AAV2/9 treatment when compared with knockout mice receiving no treatment (Fig. 5g) or WT mice (Fig. 5h).

3.5 Quantitative enzyme assay in adult tissues

Treatment with intravenous enzyme led to modest increases in PPT1 activity (23.2 – 44.9 nmol/mg/hr) in the brain compared to untreated *Ppt1*^{-/-} adults (2.5 nmol/mg/hr), but neither small nor large dose restored activity to the level seen in the brain of WT mice (215.5 nmol/mg/hr). In the heart, however, 15mg/kg of intravenous enzyme in *Ppt1*^{-/-} mice increased PPT1 activity to 132.6 nmol/mg/hr, compared to 130.7 nmol/mg/hr in WT heart (insets, Fig. 4). The lower dose resulted in a nearly undetectable difference from untreated *Ppt1*^{-/-} heart tissue. Enzyme treatment with the large dose led to a two-fold increase in PPT1 activity in *Ppt1*^{-/-} liver (401.1 nmol/mg/hr) compared to WT liver (182.7), while the lower dose increased activity to 101.9 nmol/mg/hr. Liver from untreated adult *Ppt1*^{-/-} mice contained only 5.0 nmol/mg/hr. PPT1 activity in kidney tissue from untreated *Ppt1*^{-/-} mice was very low (7.4 nmol/mg/hr) but was increased to 92.4 nmol/mg/hr with the lower dose of enzyme and 1417.4 nmol/mg/hr with the large dose of enzyme. Treatment with 15mg/kg enzyme increased PPT1 activity in the kidney of *Ppt1*^{-/-} mice nearly 9-fold compared to that seen in the kidney of WT mice (160.3 nmol/mg/hr). Finally, while the large and small doses of enzyme increased PPT1 activity in the spleen of *Ppt1*^{-/-} mice (186.0 and 29.4 nmol/mg/hr, respectively) compared to untreated *Ppt1*^{-/-} mice (7.61 nmol/mg/hr), neither increased PPT1 activity to WT level (517.3 nmol/mg/hr).

Treatment with intravenous AAV2/9-PPT1 led to higher PPT1 activity in *Ppt1*^{-/-} mice compared to untreated *Ppt1*^{-/-} mice in all tissues analyzed. PPT1 activity in the brain was slightly higher in treated *Ppt1*^{-/-} mice (6.0 nmol/mg/hr) compared to untreated mice (2.5 nmol/mg/hr). However, the treatment did not increase enzyme activity to near WT levels (215.5 nmol/mg/hr). In contrast, AAV2/9 treatment led to normal or supernormal PPT1 activity in the heart, liver, and kidney of *Ppt1*^{-/-} mice (insets, Fig. 5). In the heart, while PPT1 activity is nearly undetectable in untreated *Ppt1*^{-/-} mice, gene therapy increased it to 195.8 nmol/mg/hr, greater than that seen in WT mice (130.7 nmol/mg/hr). A similar pattern is seen in the kidney: PPT1 activity in treated mice (187.3) was greater than activity in either untreated mice (7.4 nmol/mg/hr) or WT mice (160.3 nmol/mg/hr). The greatest increase in PPT1 activity resulting from AAV2/9 treatment was observed in the liver. Untreated *Ppt1*^{-/-} mice were found to have very low liver PPT1 activity (5.0 nmol/mg/hr) compared to the levels found in WT liver (182.7 nmol/mg/hr). Treatment with gene therapy increased PPT1 activity in *Ppt1*^{-/-} mice approximately 10-fold to 1862.5 nmol/mg/hr.

4. Discussion

The goal of the current study was to evaluate a novel method for detecting and localizing PPT1 activity *in situ*. Palmitoyl protein thioesterase-1 is a lysosomal enzyme that is absent in both humans who suffer from INCL as well as the murine model of this disease. Our results indicate that we are able to detect and precisely localize PPT1 activity in mouse

tissue via histochemistry. The substrate CUS-9235 results in a reliable blue stain in those tissues that are confirmed by quantitative assay to contain PPT1 activity.

The function of PPT1 is to cleave long-chain fatty acids from cysteine residues [1]. CUS-9235 essentially mimics an endogenous fatty acid-cysteine substrate and takes advantage of PPT1's action at thioester linkages, resulting in the precipitation of a blue substrate. The method described here utilizes standard histochemical techniques and the color development chemistry is similar to the well-established β -galactosidase assay [14]. Our protocol involves using fresh frozen tissue rather than fixed tissue; following anesthetic overdose the animals were not perfused and dissected tissues were placed directly in OCT and frozen over dry ice. After trials with different fixatives we determined that 4% paraformaldehyde in PBS was the ideal fixative to use on slides prior to staining. However using a chloral-formalin acetate fixative produced similar results. Other fixatives including 1%, 3%, and 5% neutral buffered formalin, 1% and 2% paraformaldehyde, and 70% acetone were tested but led to either severely desiccated tissues or suboptimal staining. We also tested the staining protocol on tissues from animals that were perfused with 4% PFA, post-fixed for 24h in 4% PFA, then transferred to 30% sucrose. The perfusion-fixed tissue stained with similar intensity and distribution as compared with unfixed tissue. As mentioned in the methods, it is important to heed the order of addition of stain components so as to minimize precipitation. Adding components in the order listed will still result in a very fine precipitate, as indicated by the clear, pale yellow solution turning cloudy as the final component (CUS-9235) is introduced. However, this does not negatively affect staining. Future iterations of this substrate with different aliphatic chain lengths (particularly, smaller) might provide improved solubility and more sensitivity. It is important to note that this stain does not allow for quantitative measurement of enzyme activity. The infant mice presented in Figure 1 are shown at 2 $\times$ magnification and it appears that there is no blue staining in the brain of the *Ppt1*^{-/-} mouse treated with a full dose of enzyme. However, our biochemical assay reveals that levels of PPT1 in brains of infant mice treated with enzyme is actually 45% of that seen in WT mice. Upon inspection at higher magnification (20 $\times$ and 40 $\times$), blue stain becomes apparent in the brains of enzyme-treated mice, particularly in the ventricles and meninges. Though not quantitative in nature, the CUS-9235 protocol presented here does allow for the differential detection of low, intermediate, and high levels of PPT1 activity as revealed in liver, heart, and kidney tissues in Figures 4 and 5. Interestingly, it seems reasonable to speculate that even less intense or inconspicuous blue stain might correlate with decreased neuropathological markers. Two previous studies [9, 15] indicate that doses similar to ours result in relatively low brain PPT1 activity while still decreasing GFAP and CD68 and increasing lifespan. As such, highly intense blue staining may not be required to indicate therapeutic levels of PPT1 activity. Another important aspect of this protocol is maintaining the pH of tissues and staining solutions at approximately 4.5. We achieve this by using a pH 4.5 sodium acetate buffer as the base for our staining solution, and by soaking the slides in this buffer following 4% PFA fixation. Maintaining a low pH likely inactivates cytosolic thiolases that may or may not recognize and cleave our substrate.

While this study confirms that the substrate CUS-9235 and histochemical protocol described here allows us to visualize the enzyme, some interesting findings are also apparent. First is

the widespread distribution of exogenously injected PPT1 throughout the entire corpus of the neonatal *Ppt1*^{-/-} mouse, with the exception of the brain. Enzyme treatment in both infants and adults resulted in conspicuously low levels of PPT1 activity in the brain. This at least partially explains the lack of efficacy in the treatment of murine INCL using intravenous enzyme replacement alone [15] and confirms that PPT1 does not readily cross the BBB. This is not a surprising finding but indicates that while peripheral enzyme treatment may effectively treat the systemic disease associated with INCL [4], it cannot be expected to address the central nervous system aspects of the disease. However, repeated delivery of recombinant PPT1 directly to the CNS, via intrathecal or intracranial injection, could be a much more effective treatment.

Intravenous AAV2/9-mediated gene therapy to adult *Ppt1*^{-/-} mice led to broad distribution of PPT1 activity with intense staining in the kidney, liver, and heart. Other PPT1-positive organs include spleen, eye, and lung (data not shown). A surprising finding following AAV2/9-mediated gene therapy was that little or no PPT1 activity was detectable in the brain. This is in contrast to an early description of AAV9 which reported its ability to cross the BBB [16]. It is possible that the accumulation of autofluorescent material or another as yet unidentified aspect (plasma membrane perturbation, altered plasma membrane receptor expression, etc.) of PPT1 deficiency inhibits the transport of AAV2/9 across the BBB. At least one report shows that specific disease states may alter the tropism of AAV2/9 [17].

5. Conclusions

We have described a sensitive and specific method for localizing PPT1 activity in tissues. This histochemical protocol complements the current tools for the study of INCL and development of effective therapies. The method described here will be useful in evaluating treatments such as enzyme replacement, gene therapy, stem cell transplant, and small molecule drugs (suppression of non-sense mediated decay, exon-skipping or chaperones) or other future therapies that are directed at increasing PPT1 activity.

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Abbreviations

INCL	infantile neuronal ceroid lipofuscinosis
PPT1	palmitoyl protein thioesterase-1
AAV	adeno-associated virus
WT	wild-type
PBS	phosphate-buffered saline
BBB	blood-brain barrier
DMSO	dimethyl sulfoxide

PND postnatal day

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Highlights

- Infantile Batten disease is an inherited pediatric disorder characterized by deficiency in the lysosomal enzyme PPT1
- Lack of PPT1 results in intracellular accumulation of autofluorescent storage material and subsequent neuropathology
- Treatments for infantile Batten disease aim to restore PPT1 function and include enzyme replacement and CNS-directed gene therapy
- We present a novel substrate (CUS-9235) and method for localizing PPT1 activity and show results in diseased mice receiving various treatment conditions
- Results indicate that this novel protocol complements the current tools for study of the disease and evaluation of effective therapies

A. Natural substrate (S-palmitoylcysteine in peptide)

B. Histochemical substrate (5-bromo-4-chloro-3-indolyl-6-thiopalmityl-β-D-glucopyranoside)

Figure 1. Histochemical substrate for detecting PPT1 enzyme activity *in situ*

Step (1) is performed by PPT1. (A) Natural substrate for PPT1; (B) Histochemical substrate. When the indole is released by added β-glucosidase, the indole oxidizes and polymerizes in the presence of added reagents and develops a blue color.

Figure 2. Histochemical stain for PPT1 activity in newborn mice

Sagittal sections of entire newborn (PND1) mice were stained for PPT1 activity (blue) and counterstained with nuclear fast red. PPT1 activity is undetectable in untreated *Ppt1*^{-/-} mice (a) and is indicated by discrete regions of intense blue in WT mice (b). Staining of tissue in enzyme-treated *Ppt1*^{-/-} mice (c) is more uniformly blue, with the exception of the brain, indicating the presence of PPT1 activity. There also appears to be less intense staining in the retina of enzyme- treated animals compared to WT.

Figure 3. Quantitative PPT1 activity following enzyme therapy in neonatal Ppt1^{-/-} mice
Data are presented as mean ($\pm$ SEM) in nmol/mg/h, n=4 for each group.

Figure 4. PPT1 activity in adult tissues following enzyme therapy

Kidney (a–d), heart (e–h), and liver (i–l) tissues were stained for PPT1 activity (blue) using CUS-9235, then counterstained with nuclear fast red. Panels m–p show the liver at high magnification (20×) and reveal a punctate, less uniform pattern of stain in the WT liver (n) compared to the liver treated with 250 µL of enzyme (p). Arrows (→) indicate cells in the liver parenchyma, likely to be Kupffer cells, and arrowheads (►) indicate sinus-lining cells. Both cell types are revealed to contain PPT1 activity. From left to right in each row, groups are: untreated *Ppt1*^{-/-}, WT, *Ppt1*^{-/-} + 25µL enzyme, and *Ppt1*^{-/-} + 250µL enzyme. Quantitative enzyme activity is shown in the inset for each tissue and is presented as nmol/mg/hr.

Figure 5. PPT1 activity in adult tissues following AAV2/9-PPT1-mediated gene therapy
 Kidney (a–c), heart (d–f), and liver (g–i) tissues were stained for PPT1 activity (blue) using CUS-9235, then counterstained with nuclear fast red. Liver tissue at high magnification (20×) is shown in panels j–l. Arrows (→) indicate cells in the liver parenchyma, likely to be Kupffer cells, and arrowheads (▶) indicate sinus-lining cells. From left to right in each row, groups are: untreated *Ppt1*^{−/−}, WT, *Ppt1*^{−/−} + intravenous AAV2/9-mediated gene therapy. Quantitative enzyme activity is shown in the inset for each tissue and is presented as nmol/mg/hr. Note: stained sections from adult *Ppt1*^{−/−} and WT animals are the same as presented in Fig. 4. Sections displayed were chosen because they represent staining in all animals within each group.