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Decreased sensitivity of palmitoyl protein thioesterase 1-deficient neurons to chemical anoxia

Meredith Meyer¹, Attila D. Kovács^{1,2}, and David A. Pearce^{1,2,*}

¹Sanford Children's Health Research Center, Sanford Research, Sioux Falls, South Dakota, 57104 USA

²Department of Pediatrics, Sanford School of Medicine, University of South Dakota Sioux Falls, South Dakota, 57104 USA

Abstract

Infantile CLN1 disease, also known as infantile neuronal ceroid lipofuscinosis, is a fatal childhood neurodegenerative disorder caused by mutations in the *CLN1* gene. *CLN1* encodes a soluble lysosomal enzyme, palmitoyl protein thioesterase 1 (PPT1), and it is still unclear why neurons are selectively vulnerable to the loss of PPT1 enzyme activity in infantile CLN1 disease. To examine the effects of PPT1 deficiency on several well-defined neuronal signaling and cell death pathways, different toxic insults were applied in cerebellar granule neuron cultures prepared from wild type (WT) and palmitoyl protein thioesterase 1-deficient (*Ppt1*^{-/-}) mice, a model of infantile CLN1 disease. Glutamate uptake inhibition by t-PDC (L-transpyrrolidine-2,4-dicarboxylic acid) or Zn²⁺-induced general mitochondrial dysfunction caused similar toxicity in WT and *Ppt1*^{-/-} cultures. *Ppt1*^{-/-} neurons, however, were more sensitive to mitochondrial complex I inhibition by MPP⁺ (1-methyl-4-phenylpyridinium), and had significantly decreased sensitivity to chemical anoxia induced by the mitochondrial complex IV inhibitor, sodium azide. Our results indicate that PPT1 deficiency causes alterations in the mitochondrial respiratory chain.

Keywords

infantile CLN1 disease; Batten disease; *Ppt1*^{-/-}; palmitoyl protein thioesterase 1; cerebellar granule neurons; cytochrome c oxidase

1. Introduction

Neuronal ceroid lipofuscinoses (NCLs, also known as Batten disease) are recessively inherited, fatal lysosomal storage disorders with progressive neurodegeneration mostly affecting children (1). Mutations in the *CLN1* gene cause the infantile onset form of NCLs, infantile CLN1 disease (2, 3). The disease manifests between 6 and 18 months of age with developmental retardation and decreased cranial growth and typically leads to death by ages

*Corresponding author: David A. Pearce, PhD, Sanford Children's Health Research Center, 2301 E. 60th Street, Sioux Falls, South Dakota, 57104, Tel: (605)312-6004, Fax: (605)328-0401, PearceD@SanfordHealth.org.

Conflict of interest

None of the authors of this paper has any conflict of interest relating to the publication.

8-11 (3). *CLNI* encodes the soluble lysosomal enzyme, palmitoyl protein thioesterase 1 (PPT1), which removes thioester-linked fatty acyl groups such as palmitate from modified cysteine residues in proteins or peptides during lysosomal degradation (4). Although, PPT1 is ubiquitously expressed (5) and active in diverse cell types (6, 7), PPT1 deficiency causes selective neurodegeneration. Despite clues on the neuron-specific functions of PPT1 (8-12) it is still unclear why neurons are selectively vulnerable to the loss of PPT1 enzyme activity.

To examine the effects of PPT1 deficiency on several well-defined neuronal signaling and cell death pathways we compared the vulnerability of cultured wild type (WT) and *Ppt1*^{-/-} cerebellar granule neurons to different toxic insults. We found that *Ppt1*^{-/-} neurons were more sensitive to mitochondrial complex I inhibition but had significantly decreased sensitivity to chemical anoxia induced by the cytochrome c oxidase (mitochondrial complex IV) inhibitor, sodium azide (NaN₃).

2. Materials and methods

2.1 Chemicals

Neurobasal medium, B-27 neuronal serum replacement, glutamine, and penicillin-streptomycin solution were obtained from Thermo Fisher Scientific (Waltham, MA). Sodium azide, 1-methyl-4-phenylpyridinium (MPP⁺) iodide and ZnCl₂ were from Sigma Aldrich (St. Louis, MO). L-trans-pyrrolidine-2,4-dicarboxylic acid (t-PDC) was purchased from Tocris Cookson (Bristol, UK). All other chemicals unless stated otherwise were acquired from Sigma Aldrich (St. Louis, MO). The clear, polystyrene 48- and 96-well plates used for cell culture and viability assay readout were the products of Corning (Corning, NY).

2.2 Animals

Mice used in this study were wild type (WT) C57BL/6J and *Ppt1*^{-/-} (13) maintained on the same genetic background and obtained from our in house breeding colony. All experiments were carried out according to the Animal Welfare Act, NIH policies, and the guidelines developed by the Sanford Research Institutional Animal Care and Use Committee.

2.3 Cell Cultures

Primary cultures of cerebellar granule neurons were prepared from seven-day-old WT and *Ppt1*^{-/-} mouse pups as previously described (12, 14, 15). Briefly, meninges were removed from cerebella and the tissue was minced with a tissue chopper (McIlwain Tissue Chopper, Brinkmann). Minced tissue was then subjected to trypsinization and mechanical dissociation. Cultures were plated at a density of 2.25×10^5 cells per well into 48-well plates previously coated with poly-L-lysine. Cells were cultured in Neurobasal medium (supplemented to include 2% B-27 neuronal serum replacement, 25 mM KCl, 0.5 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin) and maintained in a humidified environment with an atmosphere of 5% CO₂/95% air kept at 37 °C. Culturing neurons in a serum free medium eliminates the need to add a mitotic inhibitor; in primary neuronal cultures maintained in Neurobasal/B-27, the rate of GFAP-positive glial cells is 1-2% (16). The culture medium was replaced completely approximately 24 hours after plating, and half

of the culture medium was removed and replaced every three days for the duration of time in culture.

2.4 Treatment of CGC cultures

After 14 or 21 days of *in vitro* development, cells were exposed to various concentrations of NaN_3 , t-PDC, MPP^+ and ZnCl_2 for 24 h in fresh culture medium (Neurobasal medium containing 2% B-27, 25 mM KCl, 0.5 mM glutamine, 100 U/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin). At the end of the 24-h treatments, cell viability was determined using the MTT viability assay.

2.5 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay

The MTT assay reliably measures neuronal viability in primary neuronal cultures as we have shown in several previous studies (12, 15-19). The main advantage of the MTT assay is that cell death is quantified independently of the mode of cell death (apoptosis, necrosis, atypical modes of cell death). Microscopic inspection of cultures was performed to visualize shrinkage due to apoptosis and disintegration due to osmotic lysis and thus determine approximate levels of cell death before viability was quantified using the MTT assay. The results of the MTT viability assay correlate well with what was seen in the preliminary visual inspection. The assay was performed as previously described (12, 14, 15). Briefly, at the end of the 24-h treatments, culture medium was removed and replaced with a 0.3 mg/ml solution of MTT dissolved in Neurobasal medium containing 25 mM KCl, 0.5 mM glutamine, 100 U/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin. Cells were incubated at 37 °C for 1 hour, then the medium was aspirated and the plates were let to dry. Isopropanol was added to the wells to lyse the cells and dissolve the formazan crystals. Aliquots of the dissolved formazan were then transferred to a 96-well plate, and absorbance was read at 562 nm with background subtraction at 690 nm using a SpectraMax M5 multimode microplate reader (Molecular Devices). Viability was expressed as a percentage of untreated controls.

2.6 Statistical analysis

Two-way ANOVA with Bonferroni's post-test for multiple pairwise comparisons was performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA).

3. Results

In this study, primary cerebellar granule neuron cultures prepared from WT and $Ppt1^{-/-}$ mice were exposed to various toxic insults for 24 h in order to compare the function of various signaling and cell death pathways. In neuronal cultures, the expression of neurotransmitter receptors and ion channels as well as synapse density significantly increase during *in vitro* development. Therefore, we applied the toxic treatments in our cultures at two different maturation stages, after 2 and 3 weeks *in vitro*. The toxic insults included exposures to increasing concentrations of t-PDC, MPP^+ , ZnCl_2 and sodium azide (NaN_3). Blockade of glutamate uptake by t-PDC causes highly increased extracellular glutamate levels, leading to glutamate receptor overactivation and eventually glutamate receptor-mediated cell death (20). In our WT and $Ppt1^{-/-}$ cultures, t-PDC induced the same extent of concentration-dependent neuronal cell death at both *in vitro* ages, (Fig. 1A, C).

MPP⁺, by inhibiting mitochondrial complex I, induces the generation of reactive oxygen species (ROS). In cerebellar granule neurons, the MPP⁺-induced ROS inactivate the aconitase enzyme, initiating a cascade of events with an end result of uncoupled (monomeric) nitric oxide synthase (NOS) generating more ROS and causing apoptosis (21). In 2-week-old cultures, 100 μ M MPP⁺ was significantly more toxic to *Ppt1*^{-/-} neurons than to WT neurons (**Fig. 1A**). In 3-week-old WT and *Ppt1*^{-/-} cultures, the extent of cell death induced by 100 μ M MPP⁺ was not statistically different. However, 60 μ M MPP⁺ caused markedly more cell death in *Ppt1*^{-/-} than in WT cultures (**Fig. 1C**).

Zinc enters neurons via voltage-gated calcium channels, N-methyl-D-aspartate (NMDA)-type glutamate receptors, Ca²⁺-permeable α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)- and kainate-type glutamate receptors and Zn²⁺-sensitive membrane transporters (22). High cytosolic concentration of Zn²⁺ causes mitochondrial dysfunction, increased ROS generation, mitochondrial permeability transition, cytochrome c release, and activation of voltage-gated K⁺ channels resulting in K⁺ efflux and cell shrinkage (23). Exposure to various concentrations of Zn²⁺ (50-200 μ M) induced similar amount of cell death in 2- or 3-week-old WT and *Ppt1*^{-/-} cerebellar granule neuron cultures (**Fig. 1B, D**).

Sodium azide (NaN₃) induces chemical anoxia by inhibiting the electron transfer between cytochrome c oxidase (mitochondrial complex IV) and oxygen in the mitochondrial respiratory chain. ROS generation, activation of NMDA receptors and L-type voltage-gated calcium channels, and NOS activity all contribute to the cell death caused by NaN₃ (24, 25). Surprisingly, we found that *Ppt1*^{-/-} cerebellar granule neurons were less sensitive to NaN₃-induced chemical anoxia than their WT counterparts (**Fig. 1B, D**). The effect was concentration and *in vitro* age dependent. After 2 weeks in culture, *Ppt1*^{-/-} neurons were significantly less sensitive to 100 μ M NaN₃ (**Fig. 1B**). After 3 weeks *in vitro*, 30 μ M and 50 μ M NaN₃ caused only 2% and 13% cell death in *Ppt1*^{-/-} cultures but induced 24% and 44% cell death in WT cultures (**Fig. 1D**).

4. Discussion

In this study, we found that while cultured WT and *Ppt1*^{-/-} cerebellar granule neurons were equally vulnerable to glutamate uptake inhibition-induced glutamate receptor overactivation or mitochondrial dysfunction caused by Zn²⁺ exposure, *Ppt1*^{-/-} neurons were more sensitive to mitochondrial complex I inhibition (by MPP⁺) and had significantly decreased sensitivity to chemical anoxia induced by sodium azide. In cerebellar granule neurons, MPP⁺, Zn²⁺ and sodium azide all cause mitochondria-initiated cell death with common players, such as ROS generation and NOS activity, in the cell death pathway (21, 23-25). Zn²⁺ inhibits complexes I, III and IV of the mitochondrial electron transport chain (26-28), whereas MPP⁺ specifically inhibits complex I (29, 30) and sodium azide is a specific inhibitor of complex IV (cytochrome c oxidase) (31). Our results, showing that *Ppt1*^{-/-} neurons, as compared to WT neurons, are more vulnerable to complex I inhibition (MPP⁺) but less sensitive to complex IV inhibition (sodium azide), indicate that PPT1 deficiency causes alterations in the mitochondrial respiratory chain.

Mitochondrial abnormalities have been associated with PPT1 deficiency. In fibroblasts from patients with infantile CLN1 disease, fragmented mitochondrial reticulum was observed, and reduced activities of ATP synthase and respiratory chain complexes II and IV were reported (32, 33). A recent proteomic analysis of the thalamus and cerebral cortex of symptomatic *Ppt1*^{-/-} mice found changes in the expression level of mitochondrial proteins, including decreased levels of cytochrome c oxidase subunit 7C and 2 proteins in the Fo complex of ATP synthase (34). Notably, the F1 complex of ATP synthase interacts with PPT1 (35). Furthermore, a number of putative palmitoylated proteins were identified in the mitochondrial matrix (36), indicating that PPT1 deficiency can affect the function and/or turnover of those proteins.

We have previously found that *Ppt1*^{-/-} cerebellar granule neurons in culture were significantly less sensitive to AMPA receptor-mediated toxicity but markedly more vulnerable to NMDA receptor-mediated cell death (12). In that study we used AMPA and NMDA as specific agonists of AMPA and NMDA receptors, respectively. In the present study, blockade of glutamate uptake by t-PDC induced the same extent of cell death in WT and *Ppt1*^{-/-} neurons. This finding is not surprising since the toxically increased extracellular glutamate concentration caused overactivation of both AMPA and NMDA receptors at the same time, canceling out the differences in vulnerability. Additionally, upon t-PDC exposure, glutamate also activated kainate receptors and metabotropic (G-protein-coupled) glutamate receptors, contributing to the overall toxicity and masking the differences observed previously with specific activation of AMPA or NMDA receptors.

Altogether, our results indicate that PPT1 deficiency in neurons causes functional changes in complexes I and IV of the mitochondrial electron transport chain.

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Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
DIV	day <i>in vitro</i>
MPP⁺	1-methyl-4-phenylpyridinium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCLs	neuronal ceroid lipofuscinoses
NOS	nitric oxide synthase
NMDA	N-methyl-D-aspartate
PPT1	palmitoyl protein thioesterase 1
ROS	reactive oxygen species

t-PDC	L-trans-pyrrolidine-2,4-dicarboxylic acid
WT	wild type

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Fig. 1. *Ppt1*^{-/-} cerebellar granule neurons are more sensitive to mitochondrial complex I inhibition (MPP⁺) but had significantly decreased sensitivity to chemical anoxia induced by the cytochrome c oxidase (mitochondrial complex IV) inhibitor, sodium azide

Primary cultures of cerebellar granule neurons were prepared from seven-day-old WT (solid bars) and *Ppt1*^{-/-} (open bars) mice. Cultures were maintained in Neurobasal medium with B27 serum replacement. On the 14th (A, B) or 21st (C, D) day *in vitro* (DIV 14 or DIV 21), cells were exposed to the indicated concentrations of t-PDC, MPP⁺, sodium azide (NaN₃) and ZnCl₂ for 24 h in Neurobasal medium with B27. At the end of the treatments, cell viability was determined by the MTT viability assay. Columns and bars represent mean ± SEM of experiments with 3-5 different WT and *Ppt1*^{-/-} culture preparations (n = 9-22). Statistical significance was determined by two-way ANOVA with Bonferroni's post-test for multiple comparisons.