Research article

An over-expression system for characterizing Ppt1 function in Drosophila

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Abstract

Background: The infantile onset form of Neuronal Cereoid Lipofuscinoses (INCL) is the earliest and most severe form of NCL, with neurological symptoms that reflect massive neurodegeneration in the CNS and retina. INCL is due to recessively inherited mutations at the CLN1 locus. This locus encodes the evolutionarily conserved enzyme palmitoyl-protein thioesterase 1 (PPT1), indicating an essential role for protein palmitoylation in normal neuronal function.

Results: To begin to elucidate the specific role that Ppt1 plays in neuronal cells, we have developed a Ppt1 over-expression system in Drosophila. We report that over-expression of DmPpt1 in the developing Drosophila visual system leads to the loss of cells through apoptotic cell death. This DmPpt1 over-expression phenotype is suppressed by DmPpt1 genomic deficiencies. Moreover, over-expression of DmPpt1S123A, which bears a catalytic site serine 123 to alanine mutation, does not lead to the severe eye phenotype observed with over-expression of wild-type DmPpt1. Thus, cell loss in DmPpt1 flies is directly related to the dosage of wildtype DmPpt1.

Conclusions: Although INCL is due to the loss of PPT1; increased levels of DmPpt1 also lead to neurodegeneration possibly via a detrimental effect on some aspect of PPT1’s normal function. This suggests that the precise levels of PPT1 activity are important for neuronal cell survival. The Drosophila DmPpt1 over-expression system provides a resource for genetic experiments that aim to identify the processes by which PPT1 regulates the palmitoylation-state of its essential protein substrates.

Background

The most common of the pediatric neurodegenerative diseases (1 in 12,500 births) are a set of primarily recessive disorders termed Neuronal Cereoid Lipofuscinoses (NCLs) due to the loss of central nervous system neurons and the accumulation of auto-fluorescent lipopigment [1]. While most cells contain inclusions, neurons are primarily affected leading to symptoms that include loss of vision, motor dysfunction, intellectual decline, and seizures [2]. Each NCL subtype is classified by its characteristic membrane/protein lysosomal inclusion pathology and age of onset [2]. Genetic analysis of the NCLs has identified 8 loci, CLN1-8, that are associated with the differing ages of onset of the disorders. Six of the eight loci have been mapped showing that CLN1 and CLN2 encode soluble enzymes with known functions while CLN 3, 5, 6, and 8 are putative transmembrane proteins of unknown function [2].
Infantile onset NCL (INCL) is the earliest and most severe form of NCL with symptoms that include loss of vision, motor dysfunction, intellectual decline, and seizures due to massive neurodegeneration in the CNS and retina [2]. INCL is caused by mutations in the CLN1 gene which encodes palmitoyl-protein thioesterase 1 (PPT1), suggesting that there is an important role for the regulation of palmitoylation in normal neuronal function [3]. This post-translation modification is the addition of a palmitate fatty acid chain to proteins. PPT1 is one of the enzymes that catalyze the removal of palmitoyl groups from specific protein targets. Unlike permanent fatty-acid chain additions, such as myristolation, palmitoylation is dynamic and very little is known about its role in the regulation of protein function [4].

Histochemical and biochemical analysis of PPT1 has shown that it appears to be present within the endo-lysosomal compartment and possibly in the cytoplasm [5–8]. Consistent with an important role for PPT1 in neurons, the protein is found with synaptic vesicles and synaptic vesicles in neuronal cell culture [9,10]. A model of excitotoxicity in the rat brain confirms a presynaptic localization for the protein and suggests that PPT1 may be neuroprotective during an excitotoxic event [11]. Finally, over-expression of PPT1 in a neuronal cell line protected cells from the induction of apoptosis suggesting that de-palmitoylation of p21Ras and other substrate proteins may play a role in the regulation of neuronal death [8]. While this recent work is beginning to shed light on the protein’s function, there is still little understanding of the role PPT1 plays in different cellular compartments, including tissue specific substrates and signaling pathways that it may modulate.

Drosophila has been an important model system for the study of human disease and has made important contributions to the understanding of several kinds of neuronal degeneration including Huntington’s, Parkinson’s, Alzheimer’s, and Machado-Joseph Diseases [reviewed in [12,13] and references therein]. The powerful genetic tools available in Drosophila as well as the high degree of conservation of gene function between Drosophila and higher vertebrates makes it an ideal system to study the cellular function of PPT1. The adult visual system in particular has been especially useful for characterizing human disease genes in Drosophila and for identifying modifiers of their function. In this study we present the development of an over-expression system for the study of the Drosophila Ppt1 gene.

Results

Generation of DmPpt1 Over-expression Lines

The Drosophila PPT1 homolog (DmPpt1) is ~55% identical and ~74% similar to the human protein at the amino acid level [14]. The DmPpt1 transcript appears to be expressed ubiquitously at low levels during embryonic and larval development (data not shown). Consistent with the levels of mRNA, DmPpt1 enzymatic activity is present at varying levels in all tissues that have been tested [14]. In order to produce a Drosophila model to study PPT1 function, we generated 10 independent UAS:DmPpt1 insertion lines and used the GMR-Gal4 driver line to over-express DmPpt1 in the developing visual system. The GMR Gal4 driver expresses GAL4 in all cell types including neuronal photoreceptors as the eye differentiates during the larval and pupal stages [15]. We confirmed that the UAS:DmPpt1 overexpression lines did indeed over-express DmPpt1 message by performing in situ hybridizations on third instar eye imaginal discs (data not shown). Expression of the DmPpt1 transgene was consistent with the previously described GMR GAL4 expression pattern [15]. We were not able to analyze the levels of protein produced or subcellular localization of the over-expressed protein due to the lack of a specific antibody reagent.

We analyzed the surface of the eyes in DmPpt1 over-expression adults with scanning electron microscopy (Figure 1) and at the light microscope level and found a range of morphological defects, both weak (Figure 1C) and strong (Figure 1D) depending on the UAS-DmPpt1 insertion line. The eyes show a change in size, a disruption of the individual ommatidia number and spacing as well as an absence of some sensory bristles (Figure 1A,1B,1C,1D), suggesting that there was cell loss occurring during the development of each ommatidium. Furthermore, when examined under the light microscope, the DmPpt1 over-expressing eyes contain black ommatidia (Figure 1I). These black spots do not form progressively over the life of the fly. Quantitative counting experiments over a 15 day period indicates that the number present at eclosion, while variable from fly to fly, is constant throughout the life of the individual fly (data not shown). The black spots, therefore, appear to be indicative of a degeneration event later in development once the eye has fully differentiated.

DmPpt1 Over-expression Induces Cell Death

To determine whether the underlying cellular architecture of the eye was compromised, we performed semi-thin sections on several over-expression lines, one showing a weak phenotype and one showing a strong phenotype, along with control lines (GMR:Gal4 and UAS insertion lines alone). These analyses revealed a striking loss of photoreceptors and other cell types that was correlated with the severity of the external eye morphology. Photoreceptor cell loss was quantified in the weak phenotypic line (GMR:Gal4/UAS:DmPpt1A,B,C,D) by counting individual rhabdomeres per ommatidia in semi-thin retinal sections. This quantification showed that the eyes had an average of
5.08 +/- 1.20 rhabdomeres/ommatidia (n= 266). This was compared to a GMR:Gal4/CyO control line with an average of 6.97 +/- 0.16 (n = 341) and to a UAS:DmPpt1/CyO control line with 7 rhabdomeres/ommatidia (n = 301) (Figure 1J). As indicated by the quantification, the weak phenotypic line, UAS:DmPpt18.1, showed less than the full complement of 7 rhabdomeres visible in a section of control eyes (Figure 1J). Furthermore, those photoreceptor
cells that were missing rhabdomeres appeared highly pigmented suggesting they were undergoing cell death [16]. The strong phenotypic line, UAS:DmPpt1^{8.1}, was not analyzed in this manner due to the severity of the defects and the fact that almost all rhabdomeres were missing, precluding reliable quantification.

We further analyzed the ultrastructure of the degenerating photoreceptor neurons with transmission electron microscopy (TEM) focusing on sections of the weak UAS:DmPpt1^{8.1} line, the strong UAS:DmPpt1^{2.1} line, and a control insertion alone line. TEM sections of UAS:DmPpt1 over-expression lines showed photoreceptor neurons that had become highly vacuolized with rhabdomeres in various stages of degeneration (Figure 2A,2B,2C). In a strong phenotypic line, this abnormal neuronal cell phenotype was more pronounced, with almost all ommatidia appearing highly pigmented, full of vacuoles and missing DmPpt1 over-expressing eyes undergo apoptotic cell death

Figure 2
DmPpt1 over-expressing eyes undergo apoptotic cell death. A-C, Transmission electron micrographs. A, UAS:DmPpt1^{8.1}/CyO control showing normal ommatidial structure. Scale bar = 2 µm. B, An image of GMR:Gal4/UAS:DmPpt1^{8.1} over-expression ommatidium showing individual degenerating photoreceptors (labeled with an asterisk). Scale bar = 1 µm. C, An image of GMR:Gal4/UAS:DmPpt1^{2.1} over-expression ommatidium showing all photoreceptor undergoing cell death with no rhabdomeres present. Scale bar = 1 µm. D, Scanning electron micrograph (200×) of a GMR:Gal4, UAS:DmPpt1^{8.1}/+; UAS:DmPpt1^{2.1}/+ eye. E, Scanning electron micrograph (200×) of a GMR:Gal4, UAS:DmPpt1^{8.1}/pGMR:p35 ; UAS:DmPpt1^{2.1}/+ eye.
all of their rhabdomeres. This type of ultrastructural appearance is indicative of photoreceptors undergoing programmed cell death [16].

To further test the idea that the defects we observed in eye development were the result of apoptotic cell death, we examined whether the eye morphology phenotype is altered by the presence of the baculoviral anti-apoptotic protein p35 [17]. Co-expression of p35 and DmPpt1 using the GMR promoter significantly reduced the rough eye defects associated with DmPpt1 over-expression (Figure 2D,2E). This suggests that, consistent with our TEM analysis, the defects we see are due in large part to cells dying through apoptosis.

**DmPpt1 Over-expression Mechanism**

We took two approaches to address whether the abnormal eye phenotypes are a result of increased levels of wildtype DmPpt1 or are due to an ectopic, non-wildtype function of the protein. First, two independent deficiency chromosomes that remove the genomic region containing the DmPpt1 locus were crossed into the DmPpt1 over-expression background. The rough eye defects associated with DmPpt1 over-expression (Figure 2D,2E). This suggests that, consistent with our TEM analysis, the defects we see are due in large part to cells dying through apoptosis.

In a second approach, we changed the catalytic serine at amino acid 123 to alanine (S123A) through site directed mutagenesis of the DmPpt1 cDNA. A similar mutation in the homologous amino acid in Bovine PPT1 produces an enzyme with severely reduced catalytic activity in vitro [18]. We isolated several independent UAS:DmPpt1-S123A insertion lines to test whether enzyme activity is required for the rough eye and black spot phenotypes observed with DmPpt1 over-expression. We confirmed that the transgenic lines overexpressed DmPpt1-S123A message by in situ hybridization of eye imaginal discs (data not shown). Over-expression of three UAS:DmPpt1-S123A lines with GMR-Gal4 yielded no observable abnormal phenotypes when analyzed with SEM (Figure 4A,4B). We further compared two of these lines to wild-type DmPpt1 over-expression by analyzing semi-thin sections of UAS:DmPpt1-S123A over-expressing eyes (Figure 4C,4D). For quantification, the number of rhabdomeres in each ommatidia of UAS:DmPpt1-S123A lines and the weak UAS:DmPpt1<sup>8.1</sup> phenotypic line were compared (Figure 4E). Our analysis of the two catalytic mutant lines, S123A<sup>1</sup> and S123A<sup>4</sup>, showed that they possessed 6.54 +/- 0.86 (n = 369) and 6.09 +/- 1.07 (n = 292) rhabdomeres per ommatidia, respectively. These data demonstrate that over-expression of DmPpt1 catalytic domain mutants slightly decreases the number of rhabdomeres per ommatidia.
Analysis of DmPpt1S123A catalytic mutant over-expression. A-B, Scanning electron micrographs (200×). C-D, Light microscope images of semi-thin retinal sections. Scale bar = 2 µm. A, GMR:Gal4/UAS:DmPpt1S123A^1. B, GMR:Gal4/UAS:DmPpt1S123A^4. E. Quantification of the number of rhabdomeres per ommatidia in DmPpt1S123A over-expression eyes compared to DmPpt1^8.1 over-expression eyes. *p < .00001 by Ttest.
PPT1 is involved in the de-palmitoylation of substrate proteins. This function is clearly important to neuronal cells since loss of PPT1 function leads to massive degeneration of neurons in the central nervous system of INCL patients [2] and Ppt1 knock-out mice [19]. The presence of lipofuscin and the build-up of endo-lysosomal inclusions suggest that PPT1’s function may lie in the regulated subcellular trafficking and degradation of palmitoylated proteins. While these pathological defects associated with loss of PPT1 in humans and mice have been thoroughly characterized, there is very little functional data about the specific cell biological role that this protein plays.

The protein was initially purified through its ability to de-palmitoylate p21\textsuperscript{Ras} [5]. In vitro, PPT1 has been shown to de-palmitoylate specific peptides suggesting that GAP43, the C\textsubscript{a} subunit of heterotrimeric G-proteins, and rhodopsin are possible in vivo targets [20]. Further evidence that p21\textsuperscript{Ras} may be an endogenous target was shown by the ability of over-expressed PPT1 to block apoptosis through a p21\textsuperscript{Ras}-Akt-Caspase pathway in neuroblastoma cells [8]. This inhibition was coincident with a decreased presence of p21\textsuperscript{Ras} at the membrane suggesting that PPT1 may regulate p21\textsuperscript{Ras} signaling by modulating its palmitoylation state [8]. This suggests that Drosophila Ras may be a candidate modifier of the DmPpt1 over-expression phenotype.

Conclusions
Our findings indicate that, while recessive mutations that severely decrease PPT1 cause neuronal cell death in INCL patients, increased levels of PPT1 can also lead to neurodegeneration, revealing that the precise level of PPT1 is important for neuronal cell survival. A deeper understanding of PPT1’s normal cellular function may therefore be necessary to the success of treatment strategies that aim to replace PPT1 in INCL patients. The over-expression system that we have developed in Drosophila will provide an opportunity to elucidate the role of DmPpt1 in neuronal and non-neuronal cells. The identification of genetic modifiers of this visual system phenotype will facilitate the identification of in vivo substrates and signaling pathways that DmPpt1 may modulate. The insights gained from these results will further the understanding of PPT1 function and the molecular etiology of INCL.

Methods
Fly Husbandry
All crosses were performed at 25°C on standard Drosophila media.

Transgenic Line
The full length Ppt1 cDNA was obtained from Research Genetics. It was initially identified as the EST GM14257 using the BDGP EST database. The cDNA was cloned into the EcoRI and Xhol sites of the pUAST expression vector [21]. Transgenic flies were produced by standard methods using the pP\{Wc \textaunderline{A}2,3\} helper plasmid. Transformants were identified using the white marker gene contained in the pUAST vector. We further confirmed that the transgenics lines were over-expressing DmPpt1 message through in situ hybridization on third instar eye imaginal discs. We used the GM14257 cDNA to in vitro transcribe sense and anti-sense RNAs that were then used as probes on fixed eye imaginal disc tissue.

Ppt1 mutagenesis
To produce the Serine 123 to Alanine mutation, we used PCR to change the Serine codon CTC to the Alanine codon CCG. We designed overlapping primers that contained the point mutation and used them in conjunction with two flanking primers to incorporate the mutation at Serine 123. The primers used were (the point mutation is
indicated in bold): S123A1:CGCCCTGCGCGAATC-CGATG; S123A2:CATCGGATTCGCAGGCG.

**Scanning Electron Microscopy**

Newly eclosed adults of the specified genotypes were collected and aged for several days in a yeast-free food vial. These flies were then taken through a series of ethanol dehydration steps. They were first placed in 25% ethanol and after a 12-hr incubation time they were moved to 50% ethanol. This process continued, through the following dilutions: 50, 75, 95, and 2 incubations in 100% ethanol. They were left in 100% ethanol and taken to the Northeastern Electron Microscopy facility to be critical point dried and sputter coated for scanning electron microscopy.

**Sectioning and Transmission Electron Microscopy**

Newly eclosed adults of the specified genotypes were collected, their heads were removed and then placed in a fixative solution (1% glutaraldehyde, 2% Paraformaldehyde 0.1 M Na Phosphate pH 7.4) for 1 hour at room temperature. The heads were then washed three times in 0.1 M Na Phosphate and post fixed in osmium tetroxide (2% OsO₄, 0.1 M Na Phosphate pH 7.4) for 1 hour. Following the post fixation step, the heads were washed three times for 10 minutes each in 0.1 M Na Phosphate. They were then dehydrated with an ethanol series consisting of 5 minute incubations in 30%, 50%, 70%, 85%, 95% ethanol and 2 incubations in 100% ethanol for 10 minutes each. The heads were embedded in plastic. Semi-thin plastic sections were mounted and stained with Toluidine blue. Thin EM sections were stained, mounted on grids and TEM analysis and the CBRC Drosophila Core Facility for help in generating transgenic lines. Finally we want to acknowledge the Bloomington Drosophila Stock Center for providing several stocks used in this study and the Berkeley Drosophila Genome Project for providing cDNA clones. This work was supported by NIH grant 5N033648.

**Photoreceptor Quantification**

For each genotype, the number of rhabdomeres present in each ommatidia were counted for semi-thin sections of 2 independent eyes per genotype. The following genotypes were used in the analyses: GMR:Gal4/CyO, UAS:DmPpt1$^{8.1}$/CyO, GMR:Gal4/UAS:DmPpt1$^{8.1}$, GMR:Gal4/UAS:DmPpt1S123A$^1$, and GMR:Gal4/UAS:DmPpt1S123A$^4$. All slides were blinded before scoring.

**p35 and Deficiency Suppression**

To determine the effect of p35 expression on the DmPpt1 over-expression phenotype we compared GMR:Gal4, UAS:DmPpt1$^{8.1}$/+, UAS:DmPpt1$^{2.1}$/+ (n = 12) to GMR:Gal4, UAS:DmPpt1$^{8.1}$/pGMR:p35 ; UAS:DmPpt1$^{2.1}$/+ (n = 11). To determine the effect of removing one copy of the DmPpt1 genomic locus on the over-expression phenotype, we obtained two deficiencies, Df(1)KA12 and Df(1)RA2, from the Bloomington Stock Center. We confirmed that they both removed the Ppt1 locus by performing quantitative southern blots using the DmPpt1 cDNA as a probe (data not shown). For the analysis, we collected and compare the following genotypes +/-: GMR:Gal4, UAS:DmPpt1$^{8.1}$/+; UAS:DmPpt1$^{2.1}$/+ (n = 6), Df(1)KA12/++; GMR:Gal4, UAS:DmPpt1$^{8.1}$/+; UAS:DmPpt1$^{2.1}$/+ (n = 5), and Df(1)RA2/++; GMR:Gal4, UAS:DmPpt1$^{8.1}$/+; UAS:DmPpt1$^{2.1}$/+ (n = 5). Newly eclosed adults of the specific genotypes for both experiments were collected and analyzed by SEM as described above.

**Authors’ Contributions**

CK and MM conceived of the study and designed the experiments described. CK carried out all of the experiments and drafted the manuscript. MM played an advisory role as the project developed and edited the draft manuscript. All authors read and approved the final manuscript.

**Abbreviations**

NCL, Neuronal Cereoid Lipofuscinosis; INCL, Infantile Neuronal Ceroid Lipofuscinosis; PPT1, Palmitoyl Protein-thioesterase 1

**Acknowledgements**

We would like to thank the members of the MacDonald Laboratory and Kristin White for helpful discussions during the completion of this work. We would also like to thank Bill Fowle at the Northeastern Electron Microscopy Lab for his advice and technical expertise in preparing tissue for SEM and TEM analysis and the CBRC Drosophila Core Facility for help in generating transgenic lines. Finally we want to acknowledge the Bloomington Drosophila Stock Center for providing several stocks used in this study and the Berkeley Drosophila Genome Project for providing cDNA clones. This work was supported by NIH grant 5N033648.

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