Molecular Therapy
Methods & Clinical Development
Original Article

Elevated tripeptidyl-peptidase 1 corrects multiple disease phenotypes in a mouse model of juvenile neuronal ceroid lipofuscinosis

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Juvenile neuronal ceroid lipofuscinosis (JNCL) is a neurodegenerative lysosomal storage disease caused by the loss of the endolysosomal transmembrane protein, CLN3. The main protein component of lysosomal storage material in JNCL is subunit c of mitochondrial ATP synthase (SCMAS), which is normally degraded within the lysosome by tripeptidyl-peptidase 1 (TPP1) during mitophagy. Previous studies have shown that TPP1 expression is elevated in JNCL, a potential compensatory response, while reduced levels of TPP1 exacerbate disease in a JNCL mouse model. These observations suggest a role for TPP1 in JNCL pathogenesis, and it is possible that lysosomal perturbations from the loss of CLN3 in JNCL could reduce the ability of TPP1 to degrade SCMAS. To test this hypothesis, we introduced a transgene that overexpresses TPP1 in a mouse model of JNCL and find that constitutively elevated TPP1 prevents SCMAS storage. This is associated with correction or significant reduction of other phenotypes of disease including neuroinflammation, an elevated plasma biomarker of neurodegeneration, and a disease-associated loss of brain mass with aging. From a clinical perspective, these results suggest that TPP1 augmentation could be a viable therapeutic strategy for JNCL and other lysosomal diseases that accumulate SCMAS where addressing the primary defect may be difficult or impossible.

INTRODUCTION

Neuronal ceroid lipofuscinoses (NCLs) are a group of over 12 genetically distinct but clinically related inherited neurodegenerative lysosomal storage diseases that primarily affect children and young adults.[1,2] These diseases are typically fatal, and there are no effective treatments for most. While enzyme replacement and gene therapies are feasible in principle for NCL diseases arising from defects in soluble lysosomal proteins, addressing those NCLs arising from defects in lysosomal and other transmembrane proteins continues to remain extremely challenging.

The cellular hallmark of NCLs is the accumulation of fluorescent storage material, ceroid lipofuscin, within lysosomes of neurons and other cell types.[1] For many NCLs, the major component of the storage material is subunit c of mitochondrial ATP synthase (SCMAS), a small hydrophobic protein that forms part of the pore complex.[3,4] SCMAS is located within the mitochondrial inner membrane, but during the course of mitophagy, its degradation within the lysosome is initiated by the lumenal protease, tripeptidyl-peptidase 1 (TPP1).[5] Originally suggested to play a role in pathogenesis,[6] the role of SCMAS accumulation is unclear, and there is a school of thought that it reflects rather than causes disease (reviewed in Palmer[4]).

Mutations causing a loss of TPP1 result in late-infantile NCL (LINCL),[7] and in patients, there is a rapid and extensive lysosomal accumulation of SCMAS, reflecting the critical role played by TPP1 in its degradation. However, other genetically distinct NCLs also accumulate SCMAS including juvenile NCL (JNCL). JNCL is caused by mutations in the gene *CLN3*[8] encoding an endolysosomal protein, CLN3, that may be involved in export of glycerophospholipids from the lysosome.[9] While neurons and other cells of patients with JNCL accumulate insoluble aggregates of SCMAS, they do so at a slower rate and to lower levels than in LINCL.

There is evidence to suggest a role for TPP1 in JNCL disease. Using mouse models of LINCL deficient in TPP1 ($Tpp1^{-/-}$)[10] and JNCL deficient in CLN3 protein ($Cln3^{-/-}$)[11] to investigate the effect of TPP1 genotype on disease progression in the JNCL mouse,[12] we found that heterozygosity for a *Tpp1* knockout allele has no effect on survival in an otherwise wild-type (WT) mouse, but it significantly shortens the lifespan of a $Cln3^{-/-}$ JNCL mouse. One possible explanation is that loss of CLN3 could alter the lysosomal microenvironment in such a way that partially impairs TPP1 function. This

Received 14 March 2025; accepted 2 September 2025;
https://doi.org/10.1016/j.omtm.2025.101587.

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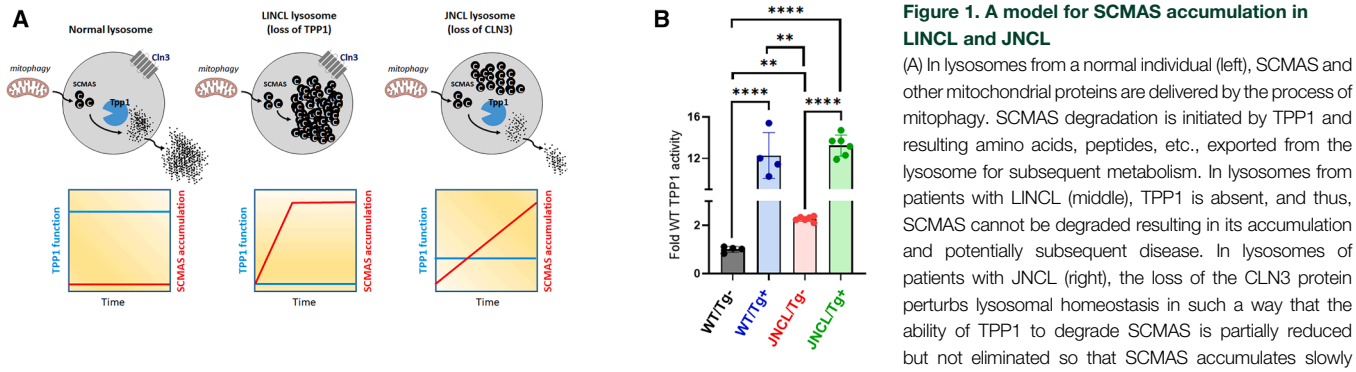


Figure 1. A model for SCMAS accumulation in LINCL and JNCL

(A) In lysosomes from a normal individual (left), SCMAS and other mitochondrial proteins are delivered by the process of mitophagy. SCMAS degradation is initiated by TPP1 and resulting amino acids, peptides, etc., exported from the lysosome for subsequent metabolism. In lysosomes from patients with LINCL (middle), TPP1 is absent, and thus, SCMAS cannot be degraded resulting in its accumulation and potentially subsequent disease. In lysosomes of patients with JNCL (right), the loss of the CLN3 protein perturbs lysosomal homeostasis in such a way that the ability of TPP1 to degrade SCMAS is partially reduced but not eliminated so that SCMAS accumulates slowly over time, correlating with the slower clinical course of JNCL compared to LINCL. (B) TPP1 activity in brain of 12-month WT and *Cln3*$^{-/-}$ mice with or without transgenic TPP1 expression. Statistical analyses were conducted by one-way ANOVA using Tukey's multiple comparisons test on log-transformed data. Note that while all possible comparisons were tested, comparison bars are only shown for significant differences. ****$p < 0.0001$; **$p < 0.01$; ns, not significant. Bars indicate mean and standard deviation.

would result in a slow accumulation of undegraded SCMAS in the lysosome (Figure 1A), reflecting or contributing to neuronal death. Consistent with this, reduction of TPP1 activity by heterozygosity for a null allele would accelerate storage and exacerbate disease. Support for this hypothesis comes from the observation that TPP1 activity is elevated in JNCL,[11–13] which may be a compensatory response to the loss of CLN3 impairing the ability of TPP1 to degrade SCMAS. If this hypothesis is correct, JNCL might be regarded, in some respects, to be a later onset and more slowly progressing form of LINCL. Consistent with this, there are patients with LINCL with hypomorphic *TPP1* mutations that resulted in slowly progressing disease, which was originally diagnosed as JNCL.[14]

If TPP1 function is impaired in JNCL, contributing to disease, we reasoned that elevating TPP1 levels could correct such deficiencies. In this report, we have tested this hypothesis using a genetic approach in which we crossed a transgenic mouse that constitutively over-expresses TPP1[15] with a *Cln3*$^{-/-}$ JNCL mouse to determine the effect of elevated TPP1 levels on SCMAS accumulation and disease progression. We find that elevated TPP1 essentially prevents SCMAS storage in the JNCL mouse in multiple tissues, and this is associated with a significant decrease in neuroinflammatory markers and correction of other phenotypes of disease.

These results indicate that TPP1 dysfunction plays a role in the pathogenesis in JNCL and provide a rationale to investigate TPP1 augmentation as a potential therapeutic strategy for patients with mutations in *CLN3*. If this is a generally applicable model for pathogenesis in lysosomal disorders that accumulate SCMAS, TPP1 augmentation may also be useful in other NCLs, especially those due to deficiencies in membrane proteins such as CLN6, CLN7, and CLN8 where correction of the primary defect remains a significant obstacle.

RESULTS

Experimental strategy

Our overall experimental strategy was to determine the effect of transgenic overexpression of TPP1 on JNCL disease progression in a mouse model. Four experimental cohorts of littermate mice were established: WT *Cln3*$^{+/+}$ animals with or without the TPP1 transgene (Tg) and *Cln3*$^{-/-}$ (JNCL) animals with or without the TPP1 Tg. For clarity, we refer to these groups as: WT/Tg+, WT/Tg−, JNCL/Tg+, and JNCL/Tg−, respectively. The TPP1 transgenic overexpresses murine TPP1 from a CAG promoter-driven Tg integrated at the ROSA26 locus. Based on enzyme activity measurements, this mouse constitutively expresses TPP1 at levels ∼10-fold and 50-fold higher than normal WT brain (Figure 1B) and liver (data not shown), respectively. In brain, expression is ubiquitous, including cortex and hippocampus.[15]

SCMAS accumulation

SCMAS storage in peripheral tissues, liver and spleen, was initially investigated at 6 months of age by immunohistochemistry (Figure 2). In WT controls (WT/Tg−) or WT animals expressing the TPP1 transgene (WT/Tg+), we observed no staining for SCMAS in liver or spleen. In contrast, JNCL mice lacking the TPP1 transgene (JNCL/Tg−) showed characteristic dark punctate inclusions of accumulated SCMAS storage material. However, JNCL mice expressing the TPP1 transgene (JNCL/Tg+) showed little or no detectable accumulation of SCMAS.

Similar results were obtained in brain (Figure 2). In JNCL/Tg− animals, storage was widespread throughout the brain with some regions of particularly intense staining, e.g., pyramidal cells of the CA3 region of the hippocampus and Purkinje cells of the cerebellum, while no SCMAS storage was detectable in control WT/Tg− and WT/Tg+ animals. Again, JNCL mice expressing the TPP1 transgene (JNCL/Tg+) showed no detectable SCMAS storage in the brain. These data indicate that in brain and peripheral organs, the accumulation of SCMAS can be prevented by increased levels of TPP1.

To determine if elimination of SCMAS is persistent, we repeated the analysis of SCMAS storage in brain at 12 months (Figure 3) in JNCL mice with or without the TPP1 Tg and WT controls (given the absence of pathology in WT mice expressing the TPP1 Tg at

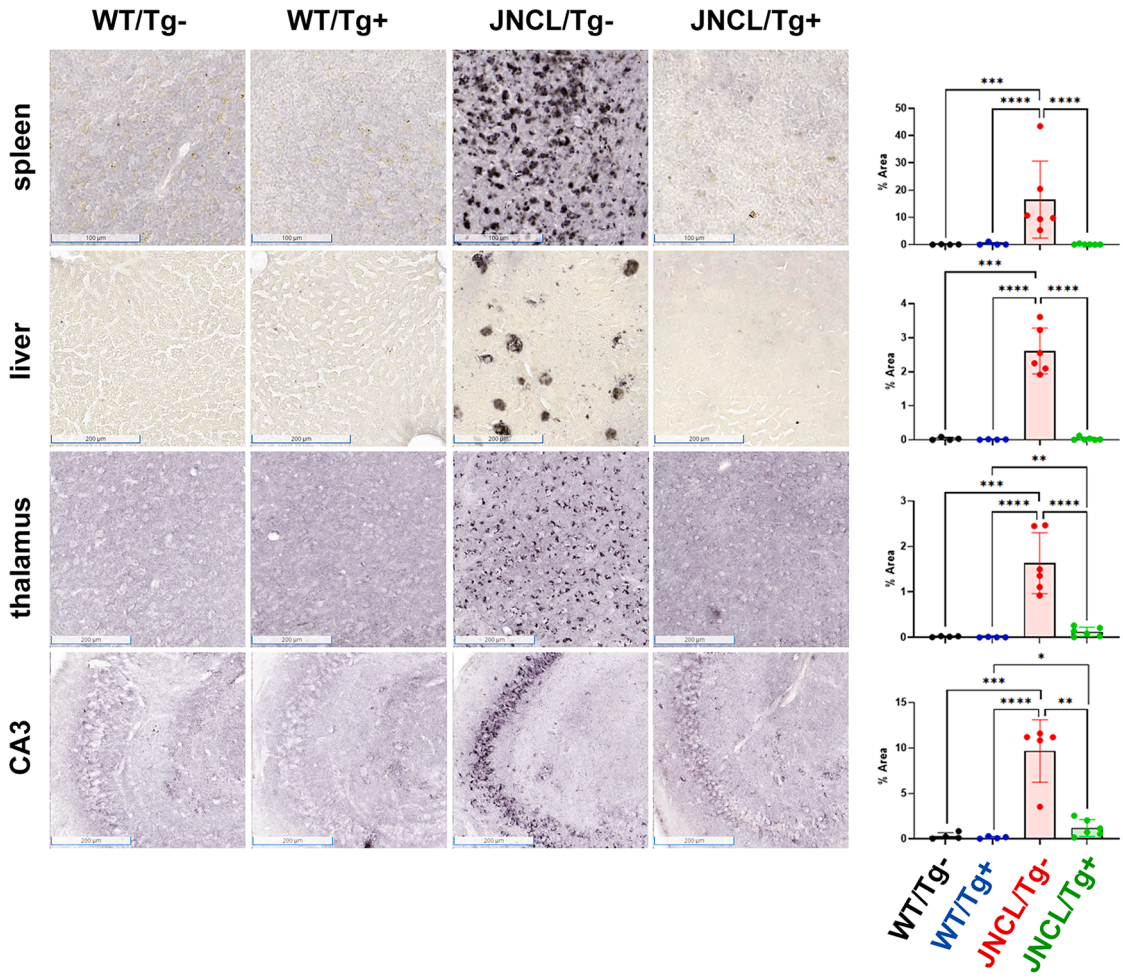


Figure 2. Elevated TPP1 prevents SCMAS accumulation in brain and peripheral tissues at 6 months

Representative images shown are from mixture of sexes as no sex-related differences in SCMAS storage were noted. Data were quantified in terms of area of image occluded by inclusions using ImageJ/Fiji. Statistical analyses were conducted by one-way ANOVA using Tukey's multiple comparisons test on log-transformed data. Note that while all possible comparisons were tested, comparison bars are only shown for significant differences. $^{****}p < 0.0001$; $^{***}p < 0.001$; $^{**}p < 0.01$, $^{*}p < 0.05$. Bars indicate mean and standard deviation.

6 months, this control was omitted in subsequent studies). Robust SCMAS storage was detected throughout the brain of the JNCL mice but not in WT controls. Consistent with findings at 6 months, expression of the TPP1 Tg essentially prevented SCMAS accumulation in the JNCL mouse.

A hallmark of NCL diseases is the autofluorescence associated with the storage material. Despite clearly preventing SCMAS accumulation, elevated TPP1 levels did not prevent autofluorescence (Figure 4), which presumably therefore arises from other components of storage material (e.g., lipids).

Neuroinflammation

Given the uncertainty regarding the role of SCMAS accumulation in disease progression, it was necessary to determine whether prevention of SCMAS storage by elevated TPP1 was associated with functional correction of disease. This goal was complicated by the extremely attenuated phenotype of the CLN3 knockout mouse.[11] For example, survival of the JNCL mouse[16,17] is only marginally truncated compared to WT controls, and while the JNCL mouse has been reported to have locomotor-behavioral defects, most reports indicate that they are nuanced and highly variable, with dramatically different data reported by different groups.[18] Six- and 12-month JNCL mice were analyzed using open field activity, RotaRod, and pole test, but animals failed to display any robust behavioral or locomotor phenotype associated with the disease (Figure S1). This was not unexpected and has been reported previously by others.[18]

As an alternative approach to measure the effect of TPP1 overexpression on disease progression in JNCL, we considered neuroinflammation. A number of recent studies (reviewed in Takahashi[19]) have

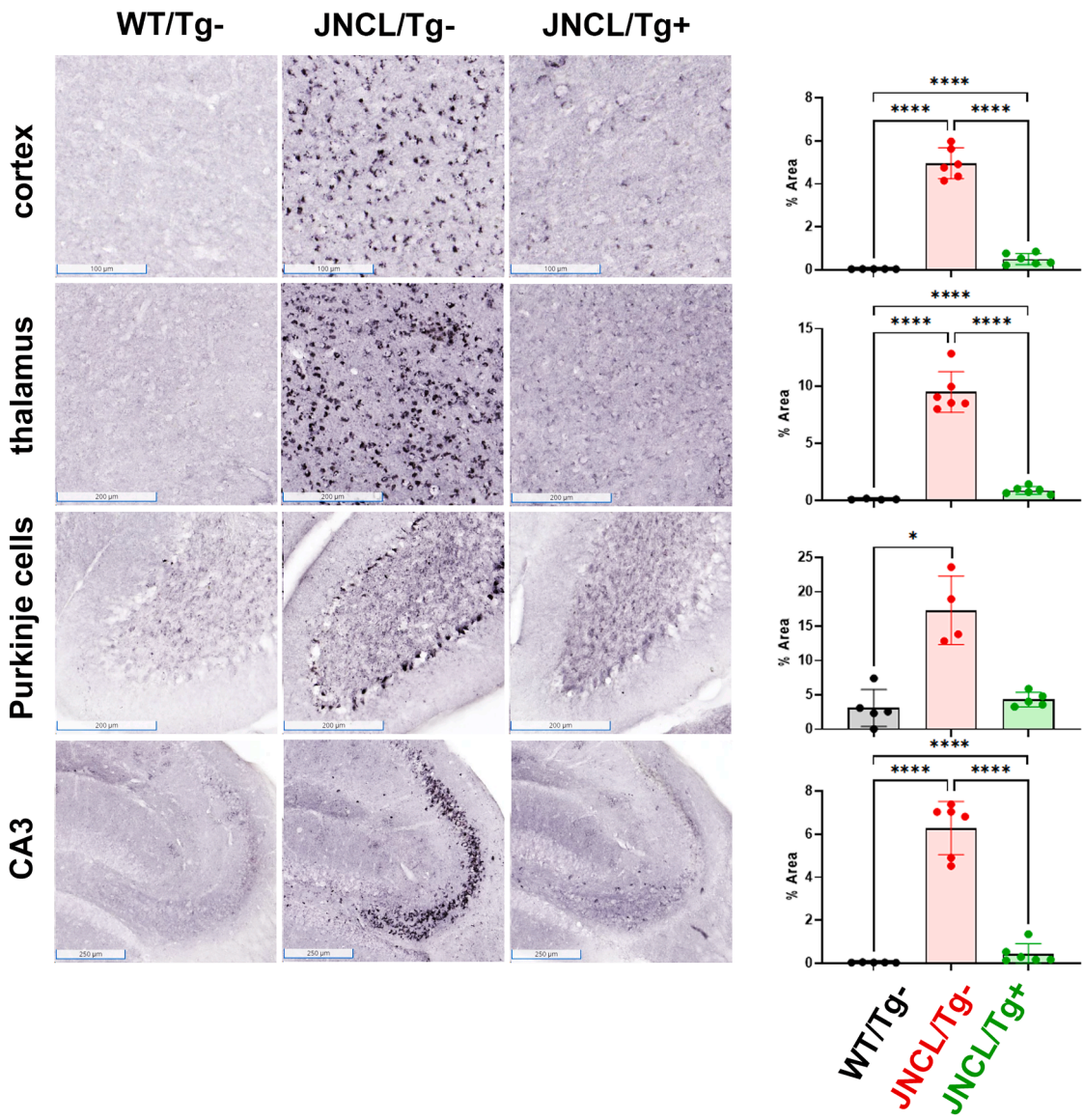


Figure 3. Elevated TPP1 prevents SCMAS accumulation in brain at 12 months

Representative images shown are from mixture of sexes as no sex-related differences in SCMAS storage were noted. Data were quantified in terms of area of image occluded by inclusions using ImageJ/Fiji. Statistical analyses were conducted by one-way ANOVA using Tukey's multiple comparisons test on log-transformed data. Note that while all possible comparisons were tested, comparison bars are only shown for significant differences. ****$p < 0.0001$, *$p < 0.05$. Bars indicate mean and standard deviation.

suggested that glial dysfunction may be a key driver of pathogenesis, and thus, therapeutically targeting both neurons and glia may provide the most successful approaches to NCL diseases. We therefore investigated the effect of TPP1 Tg expression on glial activation[20,21] in the JNCL mouse.

Activation of astrocytes was determined by immunohistochemical staining for GFAP (Figure 5). GFAP was detected throughout the brains of both JNCL and control mice, but there were several regions with JNCL-specific staining including somatosensory cortex layers 6a/b and ventral nucleus regions of the thalamus, as reported previously.[12,19] While not completely restoring GFAP staining to WT levels, expression of the TPP1 Tg resulted in a major and significant reduction in GFAP immunoreactivity in these two brain regions. Activated microglia were analyzed by immunostaining for CD68 (Figure 6). We detected sporadic CD68 staining throughout the brain of WT animals, and this was increased in the JNCL animals as reported previously.[12,19] Elevated TPP1 resulted in a minor but

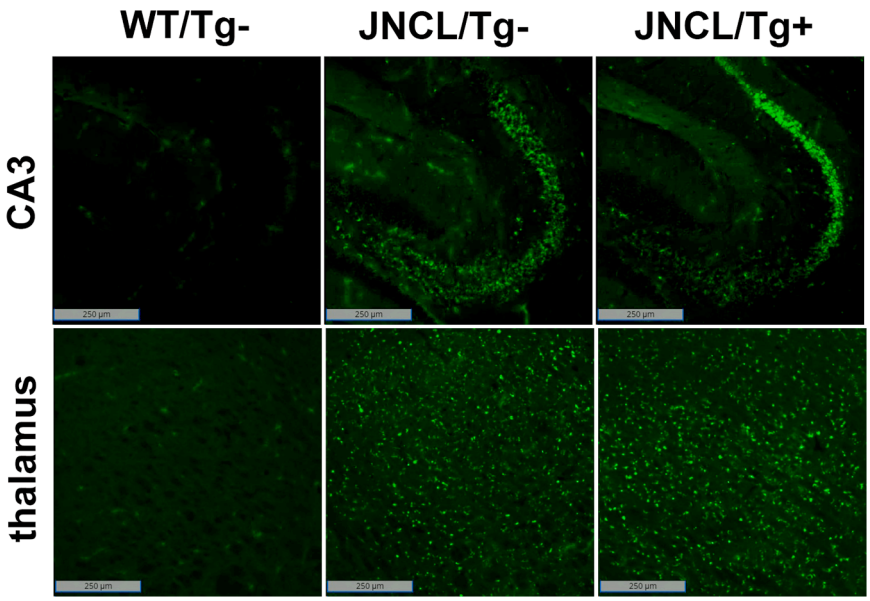


Figure 4. Elevated TPP1 does not prevent autofluorescence

Animals were 12 months old, and images were acquired from a mixture of male and females. Autofluorescence was imaged using the TRITC channel. Autofluorescence from brain sections from WT/Tg+ animals was identical to the WT control (data not shown).

significant decrease in sporadic CD68 staining observed throughout the cerebral cortex. The most extensive microglial activation in the JNCL mice was found in the ventral nuclei of the thalamus, and this was decreased significantly by elevated TPP1 levels. Taken together, these data demonstrate that TPP1 overexpression results in a significant reduction in neuroinflammation in the JNCL mouse model.

Neurofilament light

Neurofilament light (Nfl) is an established biomarker of neurodegeneration in a wide range of neurological diseases (reviewed in Yuan and Nixon[22]) including JNCL.[23,24] Nfl is a major structural protein of neurons and is released into cerebrospinal fluid and plasma in increasing amounts during neurodegeneration, inflammation, and other disease-related processes. In Figure 7, we measured Nfl by immunoassay in plasma from 12-month JNCL and WT mice, with or without transgenic TPP1 overexpression. As expected, we found that Nfl levels in plasma from the JNCL mice were significantly elevated (~2.7-fold) compared to WT animals. Expression of the TPP1 Tg had no effect on Nfl expression in WT mice, but in JNCL animals, TPP1 overexpression restored plasma Nfl to WT levels. These data suggest that elevated TPP1 reduces or prevents neurodegeneration and/or neuroinflammation in the JNCL mice.

Brain mass

Several studies[17,25] have shown that the brains of the JNCL mice are ~8% smaller in mass than age-matched WT animals at 12 months of age, presumably reflecting cortical atrophy and neuronal loss that is observed in both JNCL patients[26,27] and mice.[28] We analyzed brain weights in WT and JNCL mice, with or without transgenic TPP1 overexpression (Figures 8A and 8C). Consistent with previous studies, at ~7, 12, and 24 months, the brain mass of JNCL mice

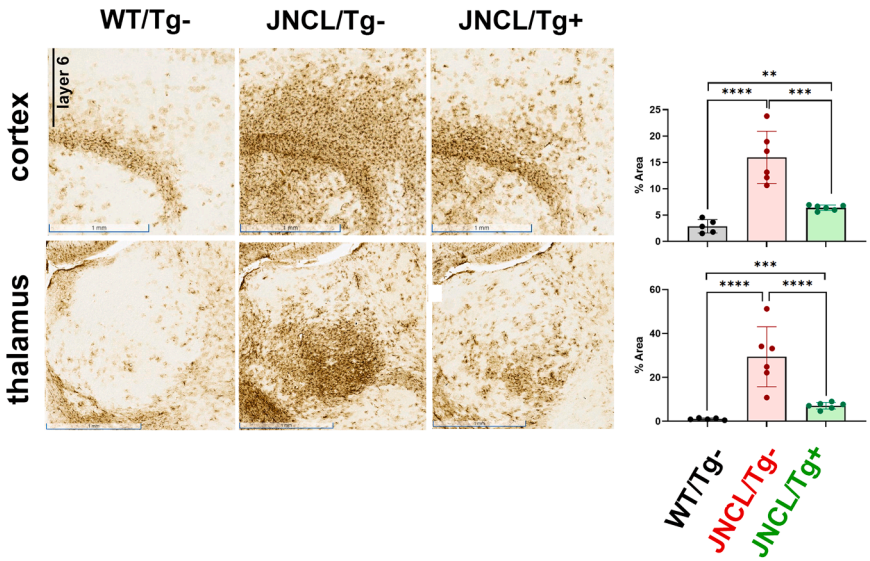


Figure 5. Increased levels of TPP1 reduce astrocyte activation in JNCL

Astrocyte activation was determined by immunohistochemical detection of GFAP. Animals are 12 months old, and images were acquired from a mixture of male and females. Regions with JNCL-specific astrocyte activation were identified as the somatosensory cortex layer 6 (upper) and ventral nuclei of the thalamus. Graphs depict percentage area occluded by GFAP staining. Statistical analyses were conducted by one-way ANOVA using Tukey's multiple comparisons test on log-transformed data. $^{****}p < 0.0001$; $^{***}p < 0.001$; $^{**}p < 0.01$. Bars indicate mean and standard deviation.

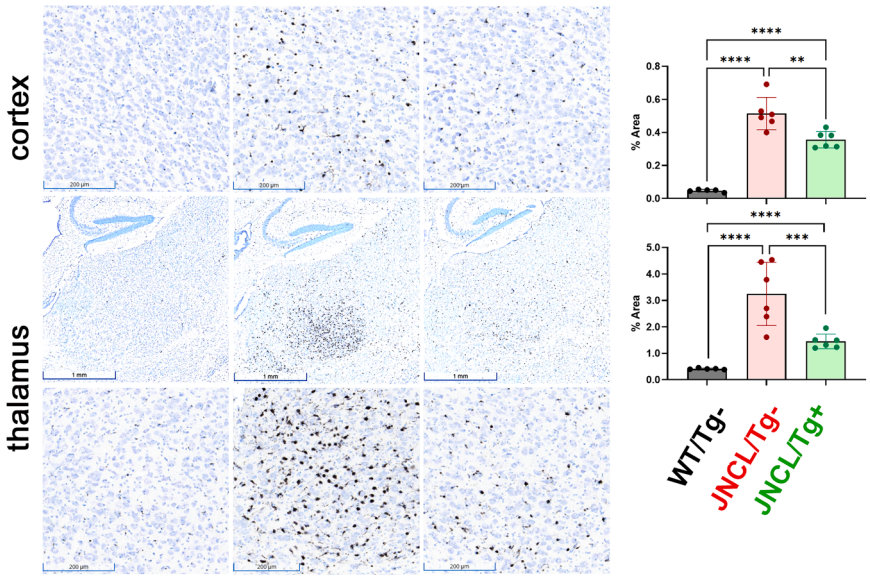


Figure 6. Increased levels of TPP1 reduce microglial activation in JNCL

Microglial activation was determined by immunohistochemical detection of CD68. Animals are 12 months old, and images were acquired from a mixture of male and females. Diffuse CD68 staining was detected throughout the cortex with images derived from the somatomotor/orbital layer 5 region. Intense JNCL-specific glial activation was identified in the ventral nuclei of the thalamus and is shown at two different magnifications. Graph indicate percentage area occluded by CD68 staining. Statistical analyses were conducted by one-way ANOVA using Tukey's multiple comparisons test on log-transformed data. Note that while all possible comparisons were tested, comparison bars are only shown for significant differences. ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$. Bars indicate mean and standard deviation.

was found to be significantly lower than that of WT animals with or without the TPP1 Tg. However, elevated TPP1 levels corrected the decrease in brain mass associated with JNCL and restored brain mass to that measured in WT mice. In contrast, liver weights in the four genotypes of animals were not significantly different (Figure 8B).

DISCUSSION

In this report, we show that elevated levels of TPP1 essentially prevent the accumulation of SCMAS, the major protein component of storage material, in cells within the brain and other tissues of the *Cln3*-knockout mouse model of JNCL. However, the role played by SCMAS in neuronal death and disease has long been a subject of debate. While defects in SCMAS degradation were originally suggested to cause NCL disease,[6] there is a school of thought that SCMAS storage and disease are not related (reviewed in Palmer[4]). However, this study clearly indicates that elimination of SCMAS in the JNCL mouse by elevated TPP1 is correlated with a partial but major reduction in neuroinflammation, which has been identified as a key driver of neuronal death.[19] In addition, transgenic TPP1 expression corrected other phenotypes of disease including elevated levels of the neurodegeneration biomarker Nfl and a progressive loss of brain mass.

It is possible that elevated TPP1 provides therapeutic benefits in JNCL independent of its role in the degradation of SCMAS; thus, lysosomal SCMAS storage may simply represent an informative biomarker for disease. Alternatively, a simpler explanation is that the accumulation of insoluble SCMAS deposits within neurons is neurotoxic and contributes to neuronal death. For example, many cellular abnormalities have been linked with JNCL,[29,30] including defects in membrane trafficking and autophagy,[31–35] and it is quite plausible that SCMAS accumulation could compromise lysosomal function, which in turn could contribute to such phenotypes. We do not suggest that SCMAS accumulation is the sole driver of disease as transgenic overexpression of TPP1 almost completely prevented SCMAS accumulation at 12 months but, while significantly reduced, some level of disease-associated neuroinflammation persisted. The pathogenic contribution of this residual neuroinflammation is difficult to evaluate, but it is worth noting that other phenotypes (Nfl and brain mass) were completely corrected. Clearly, further studies are required to definitively establish the role of TPP1 in JNCL disease and to ascertain any potential involvement of SCMAS storage.

Finally, it is worth emphasizing that discussion of whether or not SCMAS itself is a driver of neuronal death may be irrelevant from a potential translational perspective. As noted earlier, we have clearly shown that constitutively elevated TPP1 prevents SCMAS storage but also corrects multiple other phenotypes that are directly linked to disease progression.

Effect of small-molecule drugs on TPP1 function in JNCL

Several studies in *Cln3* knockout mice of small-molecule drugs that stimulate TFEB-mediated expression of lysosomal proteins, promoting lysosome biogenesis and clearance of accumulated storage material,[25,36,37] have shown promise in ameliorating disease phenotype. One of these molecules, gemfibrozil, is also thought to reduce neuroinflammation.[36] Given our findings, the therapeutic effects of inducers of TFEB-mediated transcription of lysosomal genes in JNCL mouse models may simply reflect an increase in TPP1 activity, and consistent with this, gemfibrozil treatment of JNCL mice has been shown to result in elevated TPP1.[36]

Mechanisms underlying the effects of CLN3 loss on TPP1 function

It remains to be established why the loss of CLN3 impairs TPP1 function, resulting in the lysosomal accumulation of SCMAS. Loss of CLN3 does not simply result in a downregulation of TPP1 expression or loss of catalytic function as TPP1 activity is actually elevated in

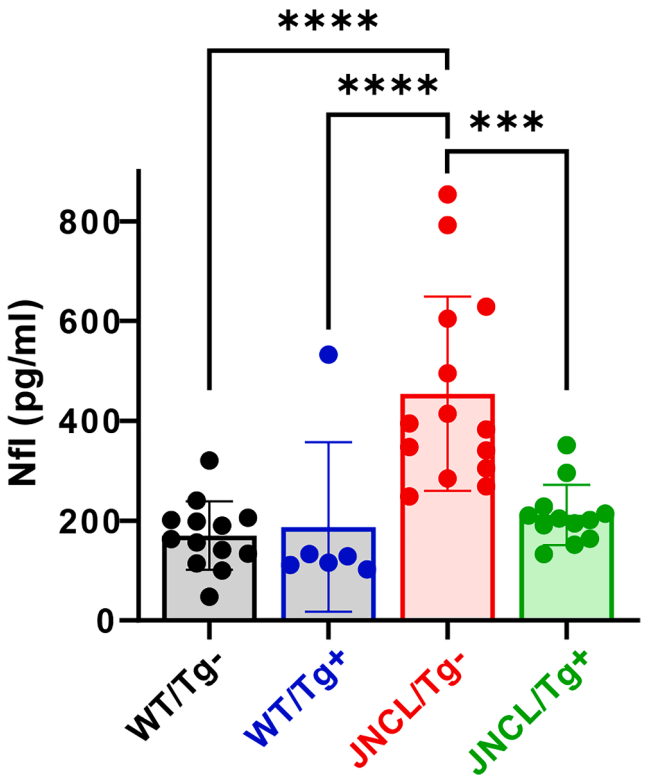


Figure 7. Elevated Nfl levels in plasma of JNCL mice are restored to WT levels by TPP1 overexpression

Statistical analyses were conducted by one-way ANOVA using Tukey's multiple comparisons test on log-transformed data. Note that while all possible comparisons were tested, comparison bars are only shown for significant differences. ****$p < 0.0001$; ***$p < 0.001$. Bars indicate mean and standard deviation. $n = 6$ animals were analyzed for WT/Tg+ and $n = 12–14$ for other genotypes.

JNCL patients and mice.[11–13] One possibility is that disturbance of the lysosomal microenvironment in the absence of CLN3 might interfere with TPP1 catalytic function (in a manner not detectable after cell lysis and processing for *in vitro* enzyme assay) or access to SCMAS. For example, a recent study[9] indicated that there is a lysosomal accumulation of glycerophosphodiesters in JNCL, which may act as inhibitors of enzymes involved in glycerophospholipid catabolism, resulting in an additional accumulation of lysophospholipids.[38] It is possible that locally elevated levels of these various lipids might inhibit TPP1, although they have no apparent effect on TPP1 activity[11] *in vitro*. Alternatively, these lipids could induce conformational changes in SCMAS that lower the rate of its degradation by TPP1. Interestingly, a mouse model of mucopolysaccharidosis III B also accumulates SCMAS even though the primary defect lies in degradation of heparin sulfate,[39] and the authors of this study speculated that "it is likely that some unfavorable condition in lysosomes slows down the degradation of SCMAS, allowing this very hydrophobic protein to pack into closely stacked arrays that may be even harder to digest."[39] Finally, it may be possible that rather than directly impairing the ability of TPP1 to degrade SCMAS, loss of CLN3 could result in increased delivery of SCMAS to the lysosome, overwhelming the capacity for TPP1 to degrade it efficiently. Of possible relevance, loss of CLN3 has been reported to interfere with mitochondrial function[31] and cellular autophagic pathways,[31,32,34,40–42] which could potentially result in increased mitochondrial turnover. Regardless of whether CLN3 loss impairs the ability of TPP1 to degrade SCMAS or increases lysosomal influx of SCMAS, increasing TPP1 levels would be predicted to reduce SCMAS storage, as we have demonstrated in this report.

Translational implications—TPP1 augmentation as a potential therapeutic route for JNCL

Universal correction of the primary defect (i.e., replacing the mutant CLN3 with a functional copy) would undoubtedly be the most effective route to therapy for JNCL. However, this remains a substantial challenge given that CLN3 is a transmembrane protein; thus, enzyme replacement therapy is not feasible, and gene therapy to correct such cell-autonomous defects continues to be challenging. Our results suggest that supplementing TPP1 levels in patients with JNCL may be a feasible alternative therapeutic strategy. While this does not address the primary defect, this approach focuses on a secondary pathogenic event that may, at least in part, drive disease progression. A major strength of this approach as a therapeutic strategy is that there are established methods to achieve TPP1 augmentation. One approach would be to conduct intracerebroventricular delivery of recombinant TPP1 in much the same way that patients with LINCL are currently treated.[43,44] However, before any possible clinical investigation, it will be necessary to demonstrate in the JNCL mouse model that delivery of recombinant TPP1 has the same therapeutic effects as constitutive transgenic overexpression, in particular, with respect to elimination of preexisting SCMAS storage. There are also continuing efforts to develop TPP1 gene therapy therapeutics for LINCL,[45] and if effective, these could also be potentially used for JNCL.

TPP1 augmentation as a general approach for lysosomal diseases that store SCMAS

In addition to LINCL and JNCL, there are other NCLs that accumulate SCMAS, including CLN5, CLN6, CLN7, and CLN8,[4] as well as a number of unrelated lysosomal storage diseases, including mucopolysaccharidosis I, II, and IIIA, multiple sulfatase deficiency, and, to variable but lesser degree, some lysosomal lipidoses.[46] If impaired TPP1 function reflected by SCMAS accumulation is a common pathogenic trigger in these diseases, then TPP1 augmentation could have application in a range of currently intractable lysosomal disorders. This is especially true for those arising from defects in transmembrane proteins, where gene and enzyme replacement therapy may be difficult or impossible and additional proof-of-principle studies using mouse or other animal models may be warranted for such disorders.

In conclusion, this study clearly demonstrates that the ability of TPP1 to degrade its substrate SCMAS is either impaired or overwhelmed in JNCL, providing a mechanistic explanation for the accumulation of this material. Importantly, we also demonstrate that elevated levels of TPP1 overcome this impairment, essentially preventing SCMAS

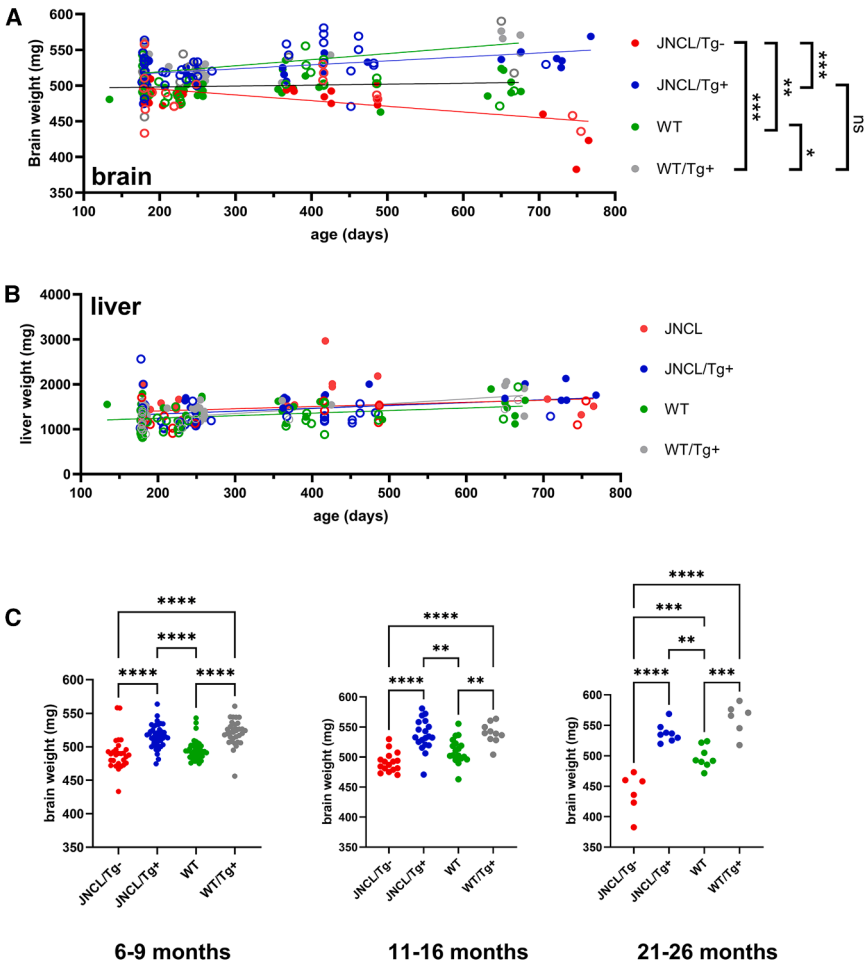


Figure 8. Progressive loss of brain mass in JNCL mice is corrected by elevated TPP1 expression

(A) Brain weights of animals are plotted against age at euthanasia. Open and closed symbols show male and female animals, respectively. Data for male and female animals were analyzed together by simple linear regression, and *p* values for all pairwise comparisons of the slopes were determined and corrected for multiple comparisons using the Bonferroni method. (B) As (A) but depicting analysis of liver weights. Pairwise comparisons of all combinations of genotypes revealed no significant differences. (C) Brain weights are compared after assigning animals into three different age groups (per genotype: n = 25–32 for 6- to 9-month group, n = 7–15 for 11- to 16-month group; n = 5–8 for 21- to 26-month group). Statistical analyses were conducted by one-way ANOVA using Tukey's multiple comparisons test on log-transformed data. All possible comparisons were tested, but unless indicated, comparison bars are only shown for significant differences. ns, not significant; ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

storage but also significantly correcting other phenotypes of disease in the JNCL mouse model. These data provide a strong rationale to consider TPP1 augmentation as a therapeutic strategy in JNCL and related diseases that accumulate SCMAS that are refractory to approaches that address the primary defect.

MATERIALS AND METHODS

Experimental design

All studies (brain weight measurements, neuropathology, behavior, and Nfl measurements) were conducted in a blinded manner where the respective researchers had access only to animal tag numbers, which convey no information regarding the genotype. Genotypes were revealed only after the collection of data. Cohort sizes of n = 6 mice were used for all studies except analysis of brain weight loss and plasma Nfl (see respective legends for details). Experimental cohorts were divided approximately equally among male and female animals.

Animals

Mice were maintained and used following protocols approved by the Rutgers University and Robert Wood Johnson Medical School Institutional Animal Care and Use Committee ("Preclinical evaluation of therapy in an animal model for LINCL," protocol I09-0274-4). Both the JNCL model[11] and the TPP1 transgenic[15] were in a C57BL/6 genetic background, and genotyping was conducted as described.[15,47] Animals were deeply anesthetized with sodium pentobarbital/phenytoin (a 1:4 dilution of Euthasol; Delmarva Laboratories, Midlothian, VA) and euthanized by transcardial perfusion with 0.9% saline for biochemical analyses. For histopathology, mice were anesthetized, perfused with saline, and then perfusion-fixed with 4% paraformaldehyde in PBS. Brains were excised, fixed for 48 h in 4% paraformaldehyde in PBS, and then transferred to 30% sucrose/PBS at 4°C until they sunk.

Neuropathology

Immunohistochemistry was conducted by NeuroScience Associates, Inc. (Knoxville, TN). In brief, mouse brains were embedded within a single block, and 35 μm sagittal sections were cut. SCMAS was visualized using an affinity-purified rabbit anti-polyclonal antibody raised against SCMAS amino-terminal peptide, which was custom-prepared by Pacific Immunology (Ramona, CA).[48] Immunohistochemistry analysis of GFAP and CD68 was conducted using rabbit anti-GFAP (DAKO) and rat anti-mouse CD68 (Bio-Rad). Sections were prepared from tissue blocks, immunostained with the primary antibodies overnight, and visualized using a biotinylated secondary antibody (goat anti-rabbit IgG, Vector Labs), followed by an avidin-biotin-HRP complex (Vector Labs) and diaminobenzidine tetrahydrochloride to create a visible reaction product as described.[49]

Measurement of Nfl

Plasma NfL concentration was measured with the Simple Plex Murine NF-L cartridge on the Ella Automated Immunoassay System according to manufacturer's instructions (Bio-techne GmbH, Minneapolis, USA). Plasma NfL results were extrapolated using the in-cartridge factory standard curve, with a quantification range of 2.7–10,290 pg/mL. Quality control (QC) samples at three concentration levels and a pooled mouse plasma sample were included in each run to monitor assay performance. Six QC samples were prepared by spiking sample diluent at 16.08, 643.13, and 6,431.3 pg/mL and spiking pooled plasma (BioIVT, NY, USA) at 16.08, 643.13, and 6,431.3 pg/mL with human/mouse Nfl control (ProteinSimple, CA, USA). Two sets of each QC were processed along with unknown samples, and the plate results were accepted if the analytical recovery of the QCs fell within the range of 80%–120% at each QC concentration. Analysis was conducted blinded to the mouse genotype.

Statistics

Data were analyzed using GraphPad Prism 10. Experimental endpoints measured were compared using ANOVA with Tukey's test for multiple comparisons using log-transformed data comparing the mean of each genotype with the mean of all other genotypes. Data are plotted on a linear scale using the adjusted p values obtained using log-transformed data. No statistical methods were used to predetermine cohort sizes.

DATA AND CODE AVAILABILITY

JNCL *Cln3*-knockout mice are available from the Jackson Laboratory (Strain# 029471) as is the parent construct for the TPP1 overexpressor, a TPP1 ROSA26 knockin mouse prior to Cre-mediated excision of the LSL stop sequence (strain# 039035).

ACKNOWLEDGMENTS

We would like to thank Dr. Robert Switzer and colleagues at NeuroScience Associates for their expert assistance with the neuropathology analyses. This study was funded by a grant from the Rutgers Health Advance program (D.E.S.).

AUTHOR CONTRIBUTIONS

Conceptualization, D.E.S. and P.L.; methodology, W.B.-P., K.E.L., E.H.S., A.B., B.S., Y. T., A.C.M., J.K.I., T.P.L., P.L., and D.E.S.; investigation, W.B.-P., K.E.L., E.H.S., A.B., Y.T., and A.C.M.; visualization, D.E.S.; funding acquisition, D.E.S. and P.L.; project administration, D.E.S. and P.L.; supervision, D.E.S., P.L., T.P.L., and B.S.; writing – original draft, D.E.S.; writing – review and editing, W.B.-P., K.E.L., E.H.S., A.B., B.S., Y.T., A. C.M., J.K.I., T.P.L., P.L., and D.E.S.

DECLARATION OF INTERESTS

D.E.S. and P.L. have received royalty payments as inventors on patents describing therapies for LINCL, the late infantile form of Batten disease, that are assigned to Rutgers University and licensed to BioMarin Pharmaceutical, Inc. The research described in this study is protected under US patent application publication no. 2024-0158772 ("Methods For Treating Lysosomal Storage Diseases," inventors: D.E.S. and P.L.).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2025.101587.

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