An experimental model of neurodegenerative disease based on porcine hemagglutinating encephalomyelitis virus-related lysosomal abnormalities

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Short report

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Abstract

Advances in experimental models for neurodegenerative diseases have enhanced the understanding of its molecular pathogenesis and begun to revealed promising therapeutic avenues. Lysosomes are involved in pathogenesis of a variety of neurodegenerative diseases and play a large role in neurodegenerative disorders caused by virus infection. However, whether virus-infected cells or animals can be used as experimental models of neurodegeneration in humans based on virus-related lysosomal dysfunction remain unclear. Porcine hemagglutinating encephalomyelitis virus (PHEV) displays neurotropism in mice and neural cells are its targets for viral progression. PHEV infection may be a risk factor for neurodegenerative diseases. Our findings demonstrated for the first time that PHEV infection can lead to lysosome disorders and showed that the specific mechanism of lysosome dysfunction is related to PGRN expression deficiency and indicated similar pathogenesis compared to human neurodegenerative diseases such as neuronal ceroid lipofuscinosis (NCL) and frontotemporal lobar degeneration (FTLD) upon PHEV infection. Trehalose can also increase progranulin (PGRN) expression and rescue abnormalities in lysosomal structure in PHEV-infected cells. In conclusion, these results suggest that PHEV may serve as a disease model for studying the pathogenic mechanisms and prevention of other degenerative diseases.

Finding

Neurodegeneration is a characteristic of many debilitating, incurable diseases that are rapidly increasing in prevalence in humans. There is an urgent need to develop new and more effective therapeutic strategies to combat these devastating diseases. Models ranging from cell-based systems to unicellular organisms and complex animals have proven to be a useful tool shedding light on the mechanisms underlying neurodegenerative diseases, and these advances have now begun to reveal promising therapeutic avenues [1]. A variety of neurotropic virus infections of the central nervous system, especially those characterized by a chronic progressive course, may produce multiple incidences of damage in infected and neighboring neurons; this damage is a possible risk factor for neurodegenerative diseases such as those caused by rabies viruses (RV) [2]and Zika virus (ZIKV) [3]. A growing body of epidemiologic and experimental data indicates that viral infections cause chronic damage resulting in alterations of neuronal function and viability by directly triggering neurotoxic pathways or the activation of immune responses. Indeed, viral agents have been reported to induce molecular hallmarks of neurodegeneration such as the production and deposition of misfolded protein aggregates, including amyloid-β (Aβ) [4]and a-synuclein (α-SYN) [5]. The questions of whether the pathogenesis of these infections shows similarity to that of human neurodegenerative diseases and whether virus-infected cells or animals can be used as experimental models of neurodegeneration in humans remain under debate.

Porcine hemagglutinating encephalomyelitis is an infectious disease of naïve pigs, in which motor disorders are often seen during field outbreaks, but the clinical disease is variable and dependent on age. This disease is caused by porcine hemagglutinating encephalomyelitis virus (PHEV), a single-stranded, nonsegmented, positive-stranded RNA coronavirus belonging to the *Betacoronavirus* genus within the

Coronaviridae family, subfamily *Cornavirina*e, order *Nidovirales* [6]. PHEV also displays neurotropism in mice and Wistar rats and produces acute encephalomyelitis [7, 8]. The results of previous *in vivo* or in *vitro* studies using mice or neuro-2a (N2a) cells have suggested that PHEV invades the central nervous system (CNS) via the peripheral nervous system (i.e., neural spread) and infects nerve cells. Typical neurological symptoms of emaciated PHEV-infected mice include generalized muscle tremors, hyperesthesia, movements of the front and hind feet similar to piano playing and a tendency to sit in a dog-like position [9]. The neuronal bodies show very little pathological change, but obvious degeneration of neuronal processes occurs after infection with pathogenic PHEV, including stunted axon elongation, unstable dendritic spine formation and disconnection of neurites [10, 11]. These observations indicate that PHEV infection may be a risk factor for neurodegenerative diseases and suggest the needs for further research on the specific mechanisms of PHEV infectivity.

Lysosomes are membrane-bound organelles that play roles in the degradation and recycling of cellular waste. Lysosomal dysfunction tends to affect the central nervous system (CNS) to a greater extent than that of other organelles because the maintenance of adequate lysosomal function is especially important for the health of postmitotic neurons in the CNS that are destined to survive the entire lifetime of the organism. Lysosomal dysfunction and defects in fusion with vesicles containing cargo are commonly observed abnormalities in proteinopathic disorders that lead to protein aggregates, which are a common pathological feature of neurodegenerative disease [12, 13]. In addition, the lysosomes are involved in the exclusion of infectious agents from the penetration of host tissue and concomitant immune regulation and must therefore be able to respond quickly with increased or decreased function to various metabolic conditions, with the aim of to protecting cells from death or damage upon virus infection [14]. Therefore, lysosomes play a large role in neurodegenerative disorders caused by virus infection. To test the roles of lysosomes in the PHEV infection process, N2a cells were infected with PHEV. The cells were fixed, and the lysosomes and PHEV were visualized by anti-LAMP1 and anti-PHEV staining. We found that PHEV localized to the lysosomes and resulted in enlarged lysosomes (Fig. 1A). Indeed, PHEV infection resulted in increased LAMP1 expression levels in *vitro* [15] and increased lysosome enlargement *in vivo* (Additional file 1). These results showed that PHEV infection leads directly to lysosomal dysfunction.

The progranulin (PGRN) protein, encoded by the granulin (*GRN*) gene, has been recently implicated in several neurodegenerative diseases [16, 17]. PGRN localizes to the lysosome and is important for proper lysosomal structure and function. Strong evidence has shown that lysosomal dysfunction occurs in cases of PGRN deficiency [18, 19]; haploinsufficiency results in frontotemporal lobar degeneration (FTLD), and complete loss results in neuronal ceroid lipofuscinosis (NCL) [20, 21]. To examine the change in PGRN expression in PHEV-infected N2a cells, cell lysates of PHEV-infected normal or EGFP-PGRN-overexpressing N2a cells were analyzed by western blotting with anti-PGRN or anti-GFP antibodies. The results showed that both endogenous and exogenous PGRN expression was obviously decreased by PHEV infection compared to mock infection (Fig. 1B, Additional file 2). Indeed, qPCR analysis also showed decreased mRNA levels of PGRN in PHEV-infected N2a cells (Fig. 1C). Furthermore, these cells were fixed, and the lysosomes, PGRN and PHEV were visualized by anti-LAMP1, anti-PGRN and anti-PHEV staining, respectively. The results showed that PHEV bound to PGRN and that PGRN was passively

transported to lysosomes because of PHEV infection (Fig. 1D, Additional file 3). To test the physical interaction between PGRN and PHEV, PHEV- or mock-infected EGFP-PGRN-transfected N2a cells were used. Cell lysates were immunoprecipitated with anti-GFP beads. A PHEV signal is detected in PHEV-infected immunoprecipitates but not under mock infection (Fig. 1E), suggesting a physical interaction between PGRN and PHEV. PGRN can be intracellularly processed into 10 kDa peptides in multiple cell types, and this processing is dependent on lysosomal activities [22]. However, we found that PGRN was not passively cleaved in the abnormal lysosomes of PHEV-infected cells (data not shown). These results showed that PHEV infection could lead to lysosome dysfunction by influencing PGRN expression and lysosome trafficking and indicated similar pathogenesis compared to human neurodegenerative diseases such as NCL and FTLD upon PHEV infection.

Furthermore, increasing evidence indicates that upregulated lysosomal function in neurons represents a promising therapeutic approach for neurodegenerative disorders by targeting lysosomal proteins and processes, particularly with small molecules and peptide drugs [23]. Recent advances in the understanding of PGRN biology emphasize its roles in lysosomal function and indicate that increasing PGRN levels is a potential therapeutic approach for multiple neurodegenerative diseases [24]. A recent study demonstrated that trehalose could upregulate progranulin expression in human and mouse models of PGRN deficiency, as a novel therapeutic strategy for treating frontotemporal dementia [25]. Therefore, we tried to verify the treatment effect of trehalose, which did not influence PGRN expression in normal cells, by using models from N2a cell-based systems after PHEV infection (Fig. 2A, 2B). Our results showed that trehalose function was successfully verified by increasing PGRN expression in PHEV-infected N2a cells (Fig. 2C). Indeed, trehalose simultaneously caused a decrease in enlarged lysosomes (Fig. 2D) and inhibited virus replication under PHEV infection (data not shown).

Conclusion

We first demonstrated that PHEV infection can lead to lysosome disorders and showed that the specific mechanism of lysosome dysfunction is related to PGRN expression deficiency. Trehalose can also increase PGRN expression and rescue abnormalities in lysosomal structure in PHEV-infected cells. The transactive response DNA binding protein (TDP-43)-associated proteinopathies related to PGRN expression have long been characterized as a main hallmark of amyotrophic lateral scleral sclerosis (ALS) and frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U, also known as FTLD-TDP). Indeed, we found that PHEV infection increased TDP-43 protein expression *in vivo* (Fig. 3) [26]. These findings are similar to the causes of several neurodegenerative diseases. Although further research is needed to shed more light on the molecular mechanisms of the neurodegenerative lesions and changes in animal behavior observed in acutely, chronically and subclinically PHEV-infected mice, our data suggest that PHEV may serve as a disease model for studying the pathogenic mechanisms and prevention of other degenerative diseases.

Materials And Methods

PHEV, PHEV-infected model, Plasmids, Antibodies and Chemical reagents-The PHEV stain used in this study was PHEV CC14 (GenBank accession number MF083115.1), which was previously procured by our research group. BALB/c mice (3 weeks old) were obtained from the Laboratory Animal Center of Jilin University. The PHEV-infected model was established in mice or N2a cells as reported previously [9, 11]. EGFP/EGFP-PGRN-overexpressing N2a cell lines were generated by our research group. The mouse anti-PHEV-S antibody was a laboratory-prepared monoclonal antibody, the rat anti-mouse LAMP1 antibody was obtained from BD Biosciences; sheep anti-GRN/progranulin antibodies were purchased from RD systems; and rabbit anti-TDP-43 antibodies, rabbit anti-CTSD antibodies and anti-GFP antibodies were procured from Proteintech. Trehalose was obtained from Sigma and was dissolved in ultrapure Milli-Q water (EMD Millipore) [25].

RT-PCR analysis-RNA was purified from cells using TRIzol reagent (Thermo Fisher Scientific). Two micrograms of total RNA was reverse transcribed using a poly(T) primer and SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). The primer sequences used in these assays are shown in Fig. 1C. qPCR was performed in a LightCycler 480 (Roche Applied Science), and transcript levels were calculated using the efficiency-adjusted $^{\Delta\Delta-CT}$ method. All transcripts were normalized to Actb [19].

Brain section and cell staining-Brain sections from PHEV-infected or mock-infected mice collected after 4 days or N2a cells collected after 48 hours were stained with anti-PHEV, anti-LAMP1, anti-PGRN, or anti-TDP43 and imaged under a confocal microscope as described previously [19].

Immunoprecipitation and western blot analysis-Cells were washed with PBS at 48 hours post-PHEV infection, and the cell lysates were collected in IP buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Triton, 0.1% deoxycholate with protease inhibitors) as described previously [10]. The lysates were immunoprecipitated using GFP-trap beads for 4 hours. Cell lysates were immunoprecipitated with anti-GFP beads. Western blot analysis was performed as described previously [27].

Abbreviations

PHEV: Porcine hemagglutinating encephalomyelitis virus；PGRN: Progranulin；GRN: Granulin；N2a: Neuro-2a；CNS: Central nervous system；RV: Rabies viruses；ZIKV: Zika virus；Aβ: A myloid-β；α-SYN: A-synuclein；NCL: Neuronal ceroid lipofuscinosis；FTLD: Frontotemporal lobar degeneration；TDP-43: The transactive response DNA binding protein

Declarations

Ethical Approval and Consent

All animal studies were conducted according to experimental practices and standards approved by the Animal Welfare and Research Ethics Committee of the College of Veterinary Medicine, Jilin University, China (permission number KT201904002), following the recommendations of the Council for International Organization of Medical Sciences on Animal Experimentation.

Consent for publication

Not applicable

Availability of supporting data

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

No potential conflict of interest was reported by the authors.

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Authors' contributions

Yungang Lan, Zi Li, and Wenqi He designed and supervised the experiments. Yungang lan, Zhenzhen Wang and Xinran Wang performed most of the tests. Gaili Wang and Kui Zhao cultured cells and analyzed immunofluorescence images.Yungang Lan drafted the manuscript, and Feng Gao revised it for valuable intellectual content. All authors read and approved the final manuscript.

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Additional Files

Additional file 1:

Immunoblot analysis of PHEV and LAMP1 *in vivo*

PHEV localizes to lysosomes, and PHEV infection results in lysosomal abnormalities. Brain sections from PHEV-infected mice or mock-infected mice were stained with anti-PHEV (green), anti-LAMP1 (red) and

Hoechst (blue). Representative images from the cortex region are shown. Scale bar=100 μm.

Additional file 2:

Western blots for exogenous PGRN

Exogenous PGRN levels were decreased in PHEV-infected EGFP-PGRN-transfected cells. Exogenous PGRN protein levels in PHEV-infected or mock-infected EGFP-PGRN-transfected N2a cells were quantified and normalized to GADPH. n=3, one-way ANOVA, *, P□0.05; Student's *t* test.

Additional file 3:

Immunoblot analysis of lysosomes, PGRN and PHEV

PGRN recruitment to lysosomes is increased by PHEV infection. PHEV-infected or mock-infected N2a cells were stained with anti-LAMP1 (red), anti-PGRN (green), anti-PHEV (Cyan) and Hoechst (blue). Pearson's overlap coefficient of LAMP1 and PGRN was calculated in PHEV-infected or mock-infected N2a cells. All the results are presented as the means ± the SD of the data from three independent experiments (*, P < 0.05). Scale bar=10 μm.

Figures

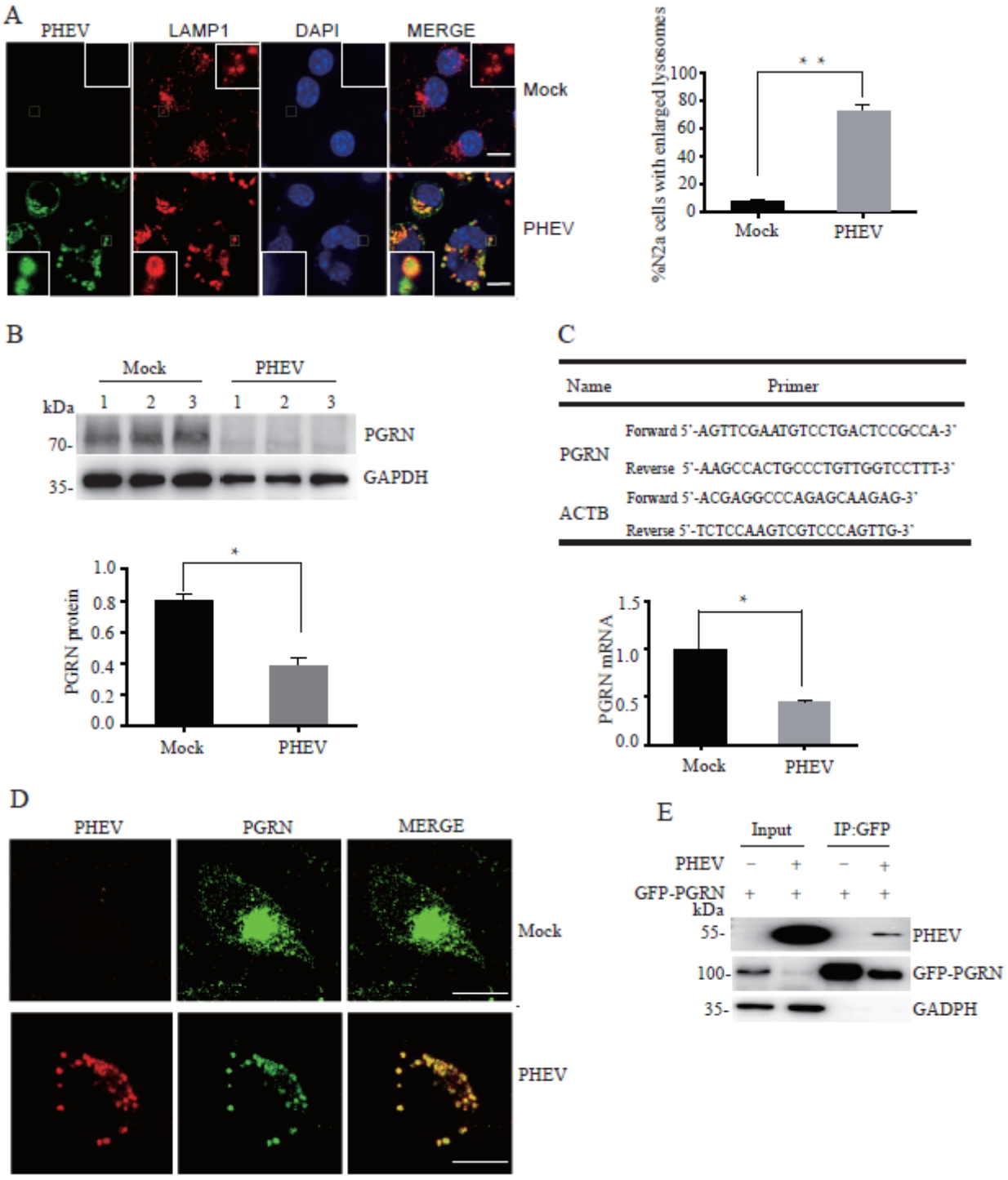

Figure 1

PHEV infection leads to lysosomal abnormalities. A. PHEV localizes to lysosomes, and PHEV infection results in greater lysosome enlargement compared to mock infection. The percentage of N2a cells containing enlarged lysosomes (≥1 μm) was quantified in the experiment. Scale bar=10 μm. All the results are presented as the means ± the SD of the data from three independent experiments (**, P < 0.01). B. PHEV infection decreased PGRN protein expression. PGRN protein levels in PHEV-infected or mock-

infected N2a cells were quantified and normalized to GADPH. n=3, one-way ANOVA, *, P□0.05; Student's t test. C. PHEV infection decreased PGRN mRNA expression. PGRN mRNA levels in PHEV-infected or mock-infected N2a cells were quantified and normalized to β-actin. n=3, one-way ANOVA, *, P□0.05; Student's t test. D. PHEV bound to PGRN. PHEV-infected or mock-infected N2a cells were stained with anti-PHEV (red) and anti-PGRN (green). Scale bar=10 μm. E. Anti-GFP immunoprecipitates from PHEV-infected or mock-infected EGFP-PGRN-transfected N2a cells were harvested, and the physical interaction between PGRN and PHEV was demonstrated.

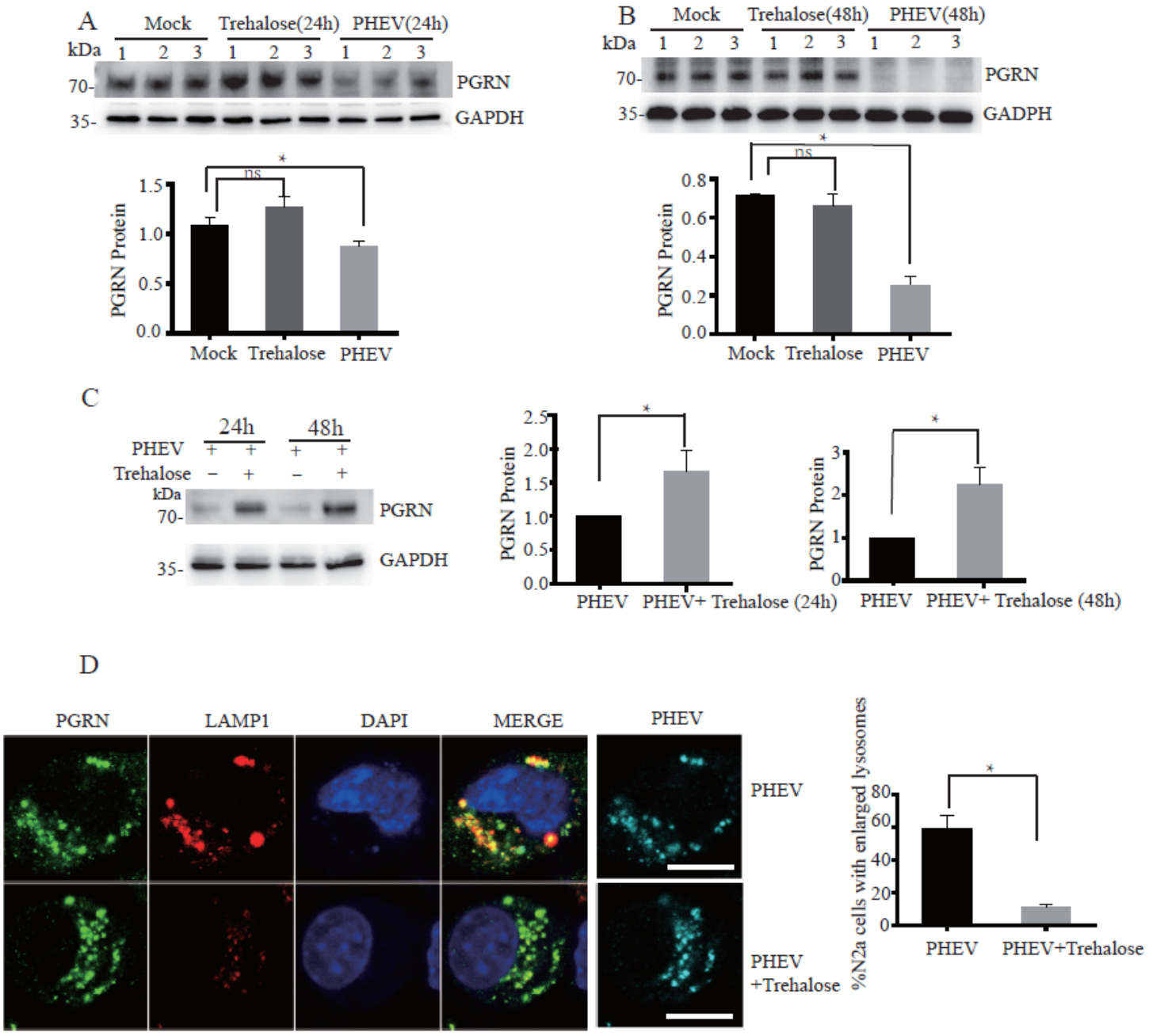

Figure 2

Trehalose increases PGRN expression and rescue abnormalities in lysosomal structure in PHEV-infected cells. A-C. Trehalose did not influence PGRN expression in normal cells but increased PGRN expression in

PHEV-infected cells. PGRN protein levels in PHEV- or mock-infected cells that were treated or not with 100 mM trehalose for 24 h or 48 h were quantified and normalized to GADPH. n=3, one-way ANOVA, *, P□0.05; Student's t test. D. There were fewer enlarged lysosomes in PHEV-infected cells treated with 100 mM trehalose compared to untreated cells treated with trehalose for 48 h. The percentage of N2a cells containing enlarged lysosomes (□1 μm) was quantified in the experiment. All the results are presented as the means ± the SD of the data from three independent experiments (*, P < 0.05). Scale bar=10 μm.

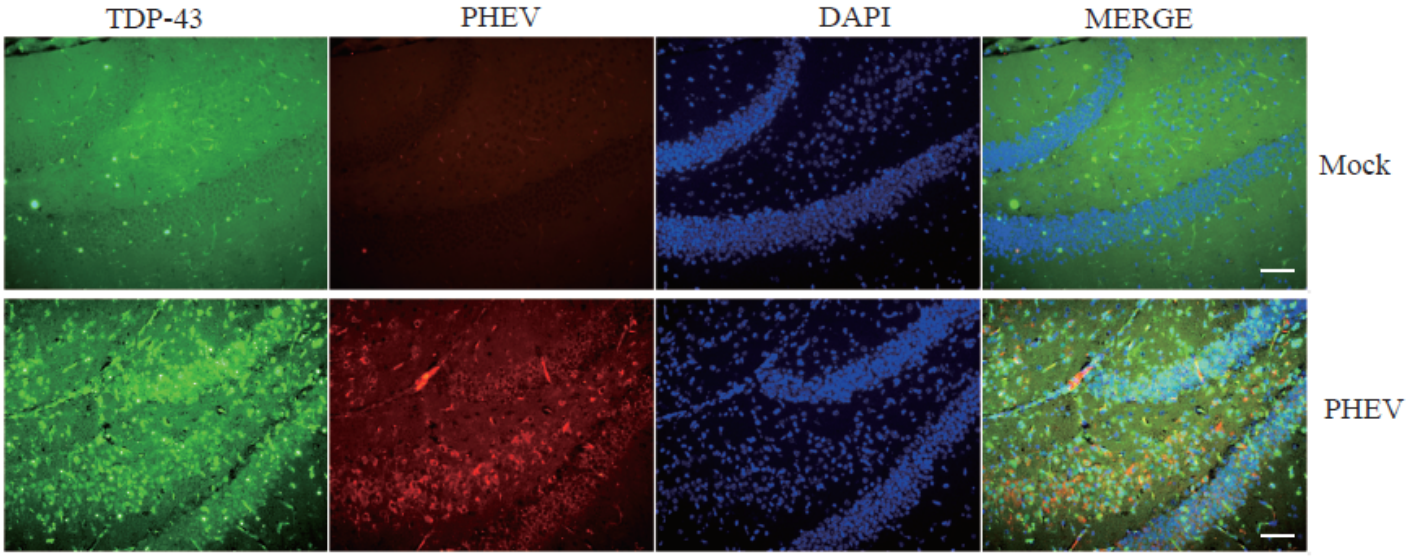

Figure 3

PHEV infection increases TDP-43 protein expression. Immunoblot analysis of PHEV and TDP-43 in vivo. Mock-infected or PHEV-infected mice were stained with anti-PHEV (red) and anti-TDP-43 (green). Cells from the hippocampus are shown as examples. Scale bar=100 μm.

Supplementary Files

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