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Phenylbutyric acid suppresses protein accumulation-mediated ER stress in retrovirus-infected astrocytes and delays onset of paralysis in infected mice

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Abstract

Many neurodegenerative diseases are associated with accumulation of misfolded proteins in cells of the central nervous system (CNS). We have previously reported that accumulation of the precursor envelope protein gPr80^{env} of *ts1*, a mutant of Moloney murine leukemia virus (MoMuLV), in the endoplasmic reticulum (ER) of infected astrocytes, results in ER stress, oxidative stress and cell death, subsequently leading to *ts1*-mediated neurodegeneration in infected mice. In the present study, we assessed whether treatments that reduce the accumulation of gPr80^{env} in the ER of *ts1*-infected astrocytes provided a protective effect against ER stress and cell death. We show that treatment with phenylbutyric acid (PBA) can prevent the unfolded protein response (UPR), ER stress and cell death in cultured *ts1*-infected astrocytes. The protective effect of PBA is associated with its ability to reduce gPr80^{env} accumulation and to increase the expression of proteins involved in protein folding in the ER, such as protein disulfide isomerase (PDI) and ERp44, rather than by decrease mRNA levels of gPr80^{env} or alter the proteasomal degradation process for gPr80^{env}. In infected mice treated with PBA we also noted a reduction in the severity of the neuropathology in brainstem tissues and a delayed onset of paralysis. These results show that PBA is a potentially effective drug for the treatment of neurodegeneration caused by protein accumulation in cells of the CNS.

Keywords

MoMuLV-*ts1*; ER stress; unfolded protein response; phenylbutyric acid; chemical chaperone

Introduction

The *ts1* mutant of the Moloney murine leukemia virus (MoMuLV) causes a progressive neurodegeneration in infected mice. This mutant contains a single point mutation in the viral *env* gene, which results in misfolding of the viral encoded precursor envelope protein gPr80^{env}. The misfolded gPr80^{env} cannot be transported efficiently from the endoplasmic

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reticulum (ER) to the Golgi compartment in astrocytes, subsequently preventing the cleavage of gPr80^{env} into the mature viral envelope proteins gp70 and PrP15E (Shikova et al. 1993; Szurek et al. 1990). In infected cells uncleaved gPr80^{env} accumulates in the ER leading to the unfolded protein response (UPR), ER stress, and oxidative stress. In *ts1*-infected mice, these events lead to CNS cell death, neurodegeneration, hindlimb paralysis and eventually death (Kim et al. 2004; Liu et al. 2004). Thus, *ts1* is a useful model to study protein accumulation associated neurodegeneration and prevention strategies.

Accumulation of misfolded proteins in the ER activates the UPR, a protective mechanism, to prevent ER stress. The major functions of the UPR are: (a) enhancing the capacity of ER folding, (b) slowing down general protein synthesis, and (c) facilitating protein degradation and trafficking out of the ER (Ron & Walter 2007). The UPR is initiated by three transmembrane ER stress sensors: the PRK-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1). In stress-free ER, these three proteins are bound by a chaperone, BiP (also known as GRP78), on their luminal domains and are held inactive (Todd et al. 2008). Upon accumulation of misfolded proteins in the ER the ER stress sensors proteins are activated. Activation of PERK induces the phosphorylation of eukaryotic initiation factor α (eIF2 α), leading to activation of activating transcription factor 4 (ATF4). These events slow down the protein translation and activate the expression of other UPR-related genes, such as ER chaperones (DuRose et al. 2009; Martinez & Chrispeels 2003; Ron & Walter 2007; Yan et al. 2008; Bonapace et al. 2004). Activation of ATF6 results in its cleavage. The cleaved cytosolic fragment translocates to the nucleus where it activates transcription of UPR-responsive genes (Malhotra & Kaufman 2007; Haze et al. 1999; Haze et al. 2001). When the amount of misfolded protein in the ER overcomes the compensatory action of the UPR, apoptotic pathways are activated including transcription factor C/EBP homologous protein (CHOP, or GADD153), which downregulates Bcl2 and activates caspase 8 (Liu et al. 2006; McCullough et al. 2001).

The ER also contains a diverse pool of molecular chaperones that aid in protein folding. Some of these chaperones, such as BiP and calregulin, directly promote proper folding and subsequent trafficking of nascent proteins (Ni & Lee 2007). Both BiP and calregulin are upregulated by the UPR. Other ER resident proteins, particularly the protein disulfide isomerase (PDI) family members also facilitate protein folding (Wang & Tsou 1993; Santos et al. 2009). The PDI family consists of PDI and other ER proteins such as ER protein 44, 57 and 72 (ERp44, ERp57 and ERp72). The specific functions of these proteins are to catalyze the scheduled formation and reduction of disulfide bonds during oxidative protein folding and to facilitate protein trafficking out of the ER (Malhotra & Kaufman 2007; Fraldi et al. 2008).

Abnormal accumulation of proteins or lipids initiates ER stress leading to many disease conditions, such as neurodegeneration, cancer, diabetes, cardiac disease and immunodeficiency (Kim et al. 2008; Matus et al. 2008; Hotamisligil 2010). Treatment with chemical chaperones has been shown to reduce protein misfolding and ER stress, resulting in a positive therapeutic effect (Balch et al. 2008; Sawkar et al. 2002). One particular chemical chaperone, phenylbutyric acid (PBA), is an orally bioavailable short-chain unsaturated fatty acid that improves ER folding capacity and trafficking. Treatment with PBA reduces ER stress (Kanki et al. 2009; Welch & Brown 1996; Ozcan et al. 2006; Ono et al. 2009; Kubota et al. 2006; Yam et al. 2007) and restores proteostasis of the cystic fibrosis transductance regulator, which is prone to misfolding. Currently PBA is being tested in clinical trials for the treatment of cystic fibrosis (Balch et al. 2008).

In previous studies, we have shown that gPr80^{env} accumulation initiates the UPR and a subsequent chain of events in the ER that causes apoptosis in *ts1*-infected astrocytes (Liu et

al. 2004; Liu et al. 2006; Kim et al. 2002; Kim et al. 2004). In fact, expression of gPr80^{env} is sufficient to induce apoptosis in gPr80^{env} transfected cells (Yu et al. 1991; Yu et al. 1997; Zhao & Yoshimura 2008) and neuropathological lesion in *ts1 env* gene transgenic mice (Yu et al. 1997). Based on the above data accumulation of gPr80^{env} is thought to be the primary insult in *ts1*-induced astrocyte dysfunction and neurodegeneration. In the present study, we investigated if PBA treatment reduces *ts1* cytopathology in cultured astrocytes and if PBA treatment delays or prevents neurodegeneration in infected mice by reducing gPr80^{env} accumulation and subsequent ER stress.

In this study, we first investigated whether PBA reduces gPr80^{env} load in *ts1*-infected astrocytes. We then determined if treatment with PBA corrects the ER stress and UPR in these cells, thus prevents *ts1*-mediated astrocyte cell death. These studies provide a mechanistic insight into the role of accumulated misfolded protein-induced neurodegeneration, and a potential mechanism by which PBA could alleviate protein misfolding in neurodegenerative disease.

Materials and Methods

Cells, cell cultures and treatment

The immortalized C1 astrocytic cell line was developed in our laboratory (Lin et al. 1997). C1 cells and NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Primary cultured astrocytes (PCA) were isolated from 1- to 2-day-old FVB/N pups as described previously (Kuang et al. 2009; Shikova et al. 1993) and grown in DMEM-F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml fungizone. The media was changed every 3 days. After four or five passages, more than 99% of the cells in the cultures were positive for the astrocyte-specific glial fibrillary acid protein (GFAP) marker. C1 cells or primary astrocytes were infected with *ts1* virus as described previously (Kuang et al. 2009). PBA (Sigma) was added to the cultures at different doses immediately after *ts1* infection, and the cells were further incubated for the time periods indicated for each experiment. Tunicamycin (Sigma) was dissolved in ethanol and was added to the cultures at a concentration of 5 µg/ml as described in the figure legend.

1.1.1.1 Western blotting analysis—C1 astrocytes, primary astrocytes and NIH3T3 cells from different treatment groups were washed with PBS and lysed in RIPA buffer as described previously (Kuang et al. 2005). Whole cell lysates were cleared by centrifugation at 13000 x g for 20 min at 4°C. Protein concentrations were determined using the Bio-Rad Dc Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA). The lysates (30–50 µg total protein per sample) were separated on SDS-PAGE gels. The proteins were then transferred to PVDF membranes, and immunoblotted with primary antibodies as described previously (Kuang et al. 2005). The primary antibodies recognize gPr80^{env} (Microbiology Associates; Burlingame, CA), cleaved caspase-3, phospho-p53, BiP, p-eIF2α, phospho-PERK, ERp44, and PDI (Cell signaling), ATF4, Bcl2, Bax, CHOP, calregulin (Santa Cruz), ATF-6α (LifeSpan Bioscience). The immunoblotted membranes were then incubated with secondary antibodies, and immune complexes were detected on the membranes using enhanced chemiluminescence (NEN Life Science Products, Boston, MA), according to the manufacturer's instructions. A monoclonal anti-β-actin antibody (Sigma) was used as a control for protein loading.

1.1.1.2 RNA extraction and real-time PCR—C1 astrocytes were infected with *ts1* at a MOI of 10, and then PBA was added at a concentration of 10 mM. After 24 or 48 h of incubation, cellular total RNA was extracted using the RNeasy kit with optional DNaseI

treatment (Qiagen, Valencia, CA). RNA was analyzed for integrity using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc. Santa Clara, CA). Total RNA (1 µg) was then used as template to synthesize cDNA with the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) and qPCR subsequently performed on the ABI 7900HT Fast Real Time PCR System (ABI) with primers specific to *ts1* gPr80^{env} using SYBR Green master mix (BioRad, Inc. Hercules, CA). RNA levels were normalized to the endogenous control gene GAPDH. Data analysis was performed using Sequence Detection System software from ABI, version 2.2.2. The experimental Ct (cycle threshold) was calibrated against the GAPDH control product. All amplifications were performed in duplicate. The DDCT method was used to determine the amount of product relative to that expressed by 24 h *ts1*-infected cells (*ts1*)-derived RNA (1-fold, 100%).

Hoechst/propidium iodide double staining for viability assay

C1 astrocytes were infected with or without PBA treatment as described above. Hoechst/propidium iodide double staining (Wise-Faberowski et al. 2004) was performed 48 h after *ts1* infection at room temperature. To identify cells with disrupted membranes, cells were firstly exposed to 40 µg/mL of propidium iodide (Roche Applied Science) dissolved in PBS for 10 min. After washing with PBS, the cells were fixed with 4% paraformaldehyde for 10 min, followed by washing with PBS, and then being treated with 10 µg/mL Hoechst 33342 (Sigma) in PBS for 4 min in the dark. After washing, the cells were stored in Hank's balanced salt solution (Invitrogen) and viewed under an inverted fluorescent microscope with the observer blinded to the treatment condition. Healthy cells were defined as those with no propidium iodide staining and without evidence of nuclear condensation. Three fields were chosen for each experimental condition and used for healthy cell counting. The average number of cells per field from uninfected control group was considered as to be 100%. The cell number counted from other groups were averaged and expressed as healthy cell % ± SEM relative to that of uninfected control group from three individual experiments.

Animals, virus inoculation and PBA treatment

FVB/N mice were obtained from Taconic Farms (Germantown, NY). The mice were divided into three groups: (a) uninfected, (b) *ts1*-infected (*ts1*), and (c) *ts1*-infected-PBA-treated (*ts1*+PBA). For infection, 3-day-old neonatal mice were injected intraperitoneally with 0.1 ml of *ts1* viral suspension containing 10⁷ of infection unit/ml per pup, as previously described (Choe et al. 1998). The *ts1*-infected-PBA-treated group (n=9) were treated with PBA intraperitoneally at 240 mg/kg daily, beginning at 5 days after birth (2 days post-infection, or dpi), until the infected untreated mice began to become paralyzed. Mice in the PBA-untreated *ts1*-infected group (n=11), or in the uninfected group were treated on the same schedule with the same volume of normal saline. All of the mice were observed carefully as signs of disease became evident, and the survival time was recorded when end-stage hindlimb paralysis appeared, as described previously (Jiang et al. 2006; Kuang et al. 2009). For histology and immunofluorescence analysis of CNS tissues, all animals were sacrificed at 30 dpi. These procedures were performed according to protocols approved by The University of Texas, MD Anderson Cancer Center Institutional Animal Care and Use Committee.

Histology and immunofluorescence analysis

Brainstem tissues frozen sections were prepared and immunostained as described previously (Kuang et al. 2009). Briefly, brainstem tissues from uninfected, *ts1*-infected untreated and *ts1*-infected PBA-treated animals were snap-frozen in Tissue-Tek OCT embedding medium (Electron Microscopy Sciences, Hatfield, PA) in liquid nitrogen, cut as 5 µm sections, and stained with hematoxylin and eosin (H&E) for histo-pathological analysis. For immunofluorescence analysis, the frozen sections were thawed, fixed in 3.7%

paraformaldehyde for 10 minutes, followed by washing and blocking 40 min in 10% donkey serum. The sections were incubated with primary antibody goat anti-gPr80^{env} overnight, and then washed three times and incubated 1 h in anti-goat IgG secondary antibody conjugated with DeLight 488 (Jackson ImmunoResearch, West Grove, PA). DAPI containing mounting medium (Invitrogen) was used to stain the nuclei. Control sections were incubated with purified goat IgGs prior to incubation in secondary antibody, or were incubated in secondary antibody alone. No nonspecific staining was observed on these control sections (data not shown).

For immunostaining of C1 astrocytes plated on chamberslides, the cells were fixed on the slides in 3.7% paraformaldehyde for 15 minutes, then washed and incubated in PBS containing 0.5% Triton X-100 for 5 min, and then in 10% donkey serum for 60 minutes. The slides were then incubated in primary antibody, which was goat anti-gPr80^{env}. After incubation, the slides were washed and incubated with anti-goat IgG secondary antibody conjugated with DeLight 488 (Jackson ImmunoResearch, West Grove, PA), followed with DAPI mounting medium.

1.2 Statistical analysis

Data are presented as means \pm SEM from three individual experiments. All cell culture experiments were conducted in triplicate. Statistical significance of the results was determined by analysis of variance (ANOVA) or Student's *t*-test. *p* value of < 0.05 was considered statistically significant. For the survival curves, the cumulative incidence of hind limb paralysis for untreated vs. PBA-treated infected mice was determined by analysis of covariance comparing slopes of curves for the two groups.

1.3 Results

PBA decreases accumulation of gPr80^{env} in *ts1*-infected cells in a dose-dependent manner

We previously reported that the envelope protein gPr80^{env} is accumulated in the ER and induces ER stress in *ts1*-infected astrocytes, leading to *ts1*-induced cytopathicity (Kim et al. 2004; Liu et al. 2004; Shikova et al. 1993; Szurek et al. 1990; Wong et al. 1992; Yu et al. 1997; Qiang et al. 2004; Qiang et al. 2006). We hypothesized that an agent that attenuated gPr80^{env} accumulation may provide a protective effect against *ts1*-induced cell death. For the current study we selected PBA because it attenuates ER stress as a chemical chaperone (Kanki et al. 2009; Kubota et al. 2006). Figure 1A shows that *ts1* infection of cultured astrocytes and NIH3T3 cells is followed by accumulation of gPr80^{env} in a time-dependent manner, but the addition of PBA can effectively decrease the accumulation of gPr80^{env} in a dose-dependent manner (both in C1 astrocytes and in NIH3T3 cells). PBA also decreases the amount of gPr80^{env} in *ts1*-infected PCAs in a dose-dependent fashion (Fig. 1B). Confirming the above results, we noted that untreated *ts1*-infected astrocytes were strongly immunoreactive for gPr80^{env}, whereas PBA-treated *ts1*-infected cells were weakly immunoreactive for gPr80^{env} (Fig. 1C). To quantify the gPr80^{env} signal, we have counted and compared the percentages of gPr80^{env} positive cell between untreated *ts1*-infected cultures and PBA-treated *ts1*-infected cultures. We found that 94.33 ± 1.202 % of untreated *ts1*-infected cells were gPr80^{env} positive, whereas only 48.57 ± 1.257 % of PBA-treated *ts1*-infected cells were gPr80^{env} positive, indicating that PBA-treated *ts1*-infected cells shown significantly less gPr80^{env} signal ($p < 0.001$).

1.3.1.1 PBA treatment does not affect mRNA levels of gPr80^{env} in infected C1 astrocytes—We sought to determine if the reduction of gPr80^{env} by PBA treatment was a result of reduced gPr80^{env} mRNA levels. The effect of PBA treatment on gPr80^{env} mRNA levels in *ts1*-infected C1 cells, was measured using real-time PCR. The results shown in

Figure 2 demonstrates that treatment of *ts1*-infected astrocytes with PBA for 24 h or 48 h did not cause significant changes in gPr80^{env} mRNA levels ($p > 0.05$). These results suggest PBA-induced reduction of gPr80^{env} protein levels in *ts1*-infected cells is not due to decreased mRNA levels of gPr80^{env}.

PBA attenuates the UPR and ER stress in *ts1*-infected astrocytes

As noted above, the UPR is initiated by activation of the ER stress sensors PERK, ATF6 and IRE1 α . To determine whether PBA can suppress UPR, we examined the effect of PBA on these ER stress sensors. Western blot analysis showed that levels of phospho-PERK (p-PERK) are upregulated in *ts1*-infected C1 astrocytes, while levels of 90KD full-length pro-ATF6 α are decreased, implying that ATF6 α protein cleavage and activation are occurring (Fig. 3A). These data indicate that the UPR is initiated in *ts1*-infected astrocytes as a consequence of gPr80^{env} accumulation in the ER. Figure 3A also shows that levels of phospho-eIF2 α (p-eIF2 α) and ATF4, downstream effectors of p-PERK, are also increased. In contrast, levels of p-PERK, p-eIF2 α , and ATF4 are all reduced in a PBA dose-dependent manner, while normal levels of full-length pro-ATF6 α are present in *ts1*-infected PBA-treated C1 cells. These results demonstrate that PBA treatment attenuates the UPR in *ts1*-infected astrocytes.

Activation of the UPR may induce transcription of UPR-responsive genes, such as ER stress markers BiP and calregulin to aid in the refolding of the misfolded proteins (Kurokawa et al. 2009). Our laboratory has previously shown that ER stress occurs in *ts1*-infected astrocytes (Kim et al. 2004; Liu et al. 2004). In light of the above findings we asked if PBA treatment affects the levels of these ER stress markers in *ts1*-infected C1 astrocytes. As shown in Figure 3B, both BiP and calregulin levels are upregulated in *ts1*-infected cells, and PBA suppresses the upregulation of BiP and calregulin. Activation of BiP in response to ER stress is generally viewed as the cytoprotective response in cells. Our results reveal a correlation between gPr80^{env} levels (Fig. 1A) and BiP or calregulin levels (Fig. 3B) at 24 and 48 hours after *ts1* infection in C1 astrocytes, suggesting that BiP or calregulin levels (ER stress markers) depend on the extent of gPr80^{env} accumulation in the cells. Interestingly, PBA reduces both gPr80^{env} (Fig. 1A) and ER stress markers (Fig. 3B) in *ts1*-infected C1 astrocytes in a similar pattern. It is very likely that the reduction of ER stress by PBA is attributed to the ability of PBA to suppress gPr80^{env} accumulation in the cells.

To further assess whether PBA affects the accumulation of other unknown misfolded proteins in the ER, we examined the effect of PBA on ER stress responses induced by tunicamycin. Tunicamycin is a known ER stress-inducer that mediates ER stress by inhibiting N-glycosylation and suppressing of protein maturation (Kubota et al. 2006). By examining the levels of the ER stress markers BiP and calregulin, we found that PBA partially attenuates tunicamycin-induced upregulation of BiP and calregulin (Fig 3C). These findings are consistent with the observation reported by Kubota et al (Kubota et al. 2006) that indicate PBA treatment inhibits tunicamycin-induced ER stress.

PBA enhances the expression of the ER folding proteins PDI and ERp44 in *ts1*-infected astrocytes

PBA treatment effectively reduced the accumulation of gPr80^{env} (Fig. 1), which lead us to ask if the reduction is due to its ability to enhance the protein-folding capacity of the ER. The ER proteins PDI and ERp44 have been shown to facilitate protein folding. We therefore compared the amounts of PDI and ERp44 in PBA-treated vs. untreated *ts1*-infected C1 astrocytes. Figure 4A-D shows that protein levels of both PDI and ERp44 are significantly decreased, in both *ts1*-infected C1 astrocytes and primary astrocytes, whereas PBA treatment was found to prevent the reduction of PDI and ERp44 levels in these cells. These

data clearly show that downregulation of ER folding proteins is accompanied with a gPr80^{env} folding deficit in *ts1*-infected astrocytes, while PBA treatment enhances the expression of ER folding proteins in these cells.

PBA treatment does not affect the proteasomal degradation process for gPr80^{env} in *ts1*-infected astrocytes

To determine whether PBA treatment prevents the accumulation of gPr80^{env} in *ts1*-infected astrocytes by accelerating its proteasomal degradation, we incubated *ts1*-infected astrocytes in 2.5 μ M of the proteasomal inhibitor MG132, following PBA treatment. MG132 treatment blocked proteasomal degradation of gPr80^{env} in *ts1*-infected PBA-untreated C1 cells, but MG132 treatment did not block the reduction of gPr80^{env} expression in *ts1*-infected PBA-treated C1 cells as shown in Figure 5. This observation suggests that PBA treatment decreases the accumulation of gPr80^{env} in infected cells by promoting the ER folding capacity or accelerating the transport of gPr80^{env}, rather than by facilitating its degradation in the PBA-treated cells.

PBA treatment prevents apoptosis in *ts1*-infected astrocytes

We have previously shown that ER stress induced by *ts1*-infection causes apoptosis of infected C1 cells (Liu et al. 2004). In light of the above results, we asked whether prevention of ER stress by PBA treatment prevents apoptosis in *ts1*-infected cells. We compared levels of ER stress activated apoptotic proteins in untreated versus PBA-treated *ts1*-infected astrocytes. Figure 6A shows that levels of CHOP, phospho-p53, Bax and cleaved caspase 3 are increased, while Bcl2 is decreased, in *ts1*-infected astrocytes, which confirms the results of previous studies (Kim et al. 2002; Kuang et al. 2005; Liu et al. 2004). In contrast, levels of CHOP, phospho-p53, Bax and cleaved caspase 3 are decreased, and levels of the anti-apoptotic protein Bcl2 are restored in PBA-treated *ts1*-infected astrocytes (Fig. 6A). The phase contrast images of astrocytes in Figure 6B and the hoechst 33342/propidium iodide double staining viability data in Figure 6C, taken together show that PBA treatment prevents cell death in *ts1*-infected astrocytes. This result is consistent with previous work from our laboratory showing that PBA promotes survival of *ts1*-infected astrocytes (Liu et al. 2002).

PBA treatment delays the onset of paralysis in *ts1*-infected FVB/N mice

We administered PBA to *ts1*-infected FVB/N mice to determine whether PBA treatment of *ts1*-infected mice decreases CNS accumulation of gPr80^{env}, and to find out if PBA can prevent *ts1*-induced neurodegeneration. The survival curves in Figure 7A show that untreated *ts1*-infected (*ts1* only) mice developed paralysis at 35 dpi, whereas paralysis is significantly delayed in PBA-treated *ts1*-infected (*ts1*-PBA) mice. In brainstem tissue sections stained with H&E, uninfected mice show normal neuropil cytological structures, whereas sections from the same region of *ts1*-infected CNS tissues contain large spongiform lesions. However, brainstem sections from infected, PBA-treated mice have nearly normal neuropil structure, without discrete spongiform foci (Fig. 7B). To determine whether PBA treatment reduces the accumulation of gPr80^{env} in CNS tissues of infected mice, we immunostained the same brainstem sections with anti-gPr80^{env} antibody. Figure 7C shows that gPr80^{env} staining was present and widespread in *ts1*-infected untreated brainstems, while few cells were stained with gPr80^{env} in sections from brainstems of PBA-treated *ts1*-infected mice. This result is consistent with our in vitro data shown in Figure 1C, indicating that PBA treatment reduces gPr80^{env} accumulation.

1.4 Discussion

Many neurodegenerative conditions are associated with protein misfolding, protein accumulation and ER stress in CNS cells (Matus et al. 2008; Kim et al. 2008; Scheper &

Hoozemans 2009; Winklhofer et al. 2008; Hashimoto et al. 2003; Hoozemans et al. 2006; Tan et al. 2009; Lindholm et al. 2006; Ono et al. 2009; Inden et al. 2007; Kubota et al. 2006; Castilla et al. 2004; Hetz & Soto 2006; Ilieva et al. 2007; Kanekura et al. 2009). When the early cytoprotective machinery (the UPR or antioxidant defenses) fails to correct protein misfolding or protein accumulation in CNS cells, apoptotic pathways are activated (Boyce & Yuan 2006; Zhang & Kaufman 2008a; Zhang & Kaufman 2008b). Thus, approaches that minimize or correct protein misfolding are important for the treatment of neurodegeneration associated with the accumulation and aggregation of misfolded proteins. In the present study, we have identified that accumulation of the gPr80^{env} protein in *ts1*-infected astrocytes induces the UPR, ER stress and apoptosis, and that the chemical chaperone PBA ameliorates the death of infected astrocytes and delays the onset of paralysis in *ts1*-infected mice by reducing the accumulation of gPr80^{env} in infected astrocytes.

Using real-time PCR, we demonstrate that *ts1* gPr80^{env} mRNA levels are not altered with PBA treatment in *ts1*-infected astrocytes. The experiments employing the proteasomal degradation inhibitor MG132 also showed that PBA does not exert its effects by promoting proteasomal degradation of gPr80^{env}. Furthermore, PBA suppresses ER stress induced by tunicamycin, which prevents protein transport from the ER to the Golgi through inhibiting protein N-glycosylation, resulting in protein accumulation in the ER. Taken together these data suggests that PBA suppresses *ts1*-induced ER stress and reduces the amount of misfolded protein, including gPr80^{env}. PBA likely acts as a chemical chaperone to enhance the capacity of ER protein folding, rather than to reduce *ts1* gPr80^{env} mRNA levels or to promote proteasomal degradation of gPr80^{env}. Further support for PBA acting as a chemical chaperone is documented in a number of recent reports (Bonapace et al. 2004; Kubota et al. 2006; Sawkar et al. 2002; Kanki et al. 2009; Inden et al. 2007; Ozcan et al. 2006; Ono et al. 2009).

The ER proteins PDI and ERp44, which are associated with protein folding, are maintained at normal levels in PBA-treated infected cells, whereas the levels of these proteins are significantly reduced in untreated *ts1*-infected cells. We suggest two possible mechanisms for these events. First, PDI is cleaved by caspase 3 and caspase 7 during apoptosis (Na et al. 2007), subsequently *ts1*-infection-induced activation of caspase 3 may lead to cleavage of PDI, thus reducing its levels and its availability for preventing of gPr80^{env} accumulation. Second, accumulation of misfolded gPr80^{env} may exhaust available levels of PDI and ERp44 during the process of disulfide bond formation or folding. Regardless of mechanisms, the data presented in this study show that decreased levels of PDI and ERp44 correlate with gPr80^{env} accumulation and that the effect of PBA in maintaining the levels of PDI and ERp44 is likely to be associated with its ability to decrease gPr80^{env} accumulation. We noted that BiP and calregulin levels correlates with gPr80^{env} in *ts1*-infected astrocytes in presence or absence of PBA treatment. BiP has been shown to bind to the precursor envelope protein of another neurovirulent murine retrovirus, suggesting its function as ER chaperone (Portis et al. 2009). Overexpression of BiP prevents activation of ER stress transducer (Bertolotti et al. 2000). These further support the notion that BiP plays a protective role under ER stress. The mechanisms for the opposite regulation of PDI and ERp44 with respect to BiP and calregulin in *ts1*-infected cells are unclear at present and need further investigation. We suspect that PDI and ERp44 chaperones are regulated by distinct pathways of the UPR compared to the regulation of BiP and calregulin following *ts1* infection and PBA treatment.

Based on this study and previous studies generated by our laboratory, we believe that PBA may play multiple roles against *ts1*-induced neurodegeneration. We have shown that PBA is a peroxisome proliferator receptor promoter and that this activity increases levels of catalase, an enzyme that converts hydrogen peroxide to water and oxygen (Liu et al. 2002).

Among these effects, decreasing the accumulation of gPr80^{env} is likely the primary mechanism associated with its neuroprotective effects. Since oxidative stress can activate ER stress and vice versa, a treatment that targets one of these effects may also alleviate the other. This hypothesis is supported by other studies showing that reactive oxygen species can be produced by the accumulation of misfolded proteins, and that antioxidants reduce ER stress and improve protein secretion, thereby reducing protein accumulation (Malhotra et al. 2008). We have also previously demonstrated that treatment of *ts1*-infected mice with the antioxidant compound monosodium luminol (GVT or MSL) extended their survival by decreasing gPr80^{env} accumulation in T cells (Scofield et al. 2009a; Scofield et al. 2009b) and astrocytes (Kuang X & Wong PK unpublished data). This may be due to the fact that GVT, in addition to acting as a free radical scavenger, also upregulates the cellular antioxidant system with activation and nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2) (Reddy et al. 2010). These studies suggest that PBA and GVT may share common mechanisms responsible for ameliorating the effects of gPr80^{env}. In both cases, CNS and T cell protection may be associated with the prevention of gPr80^{env} accumulation and the accompanying prevention of ER and oxidative stress.

Among known chemical chaperones, PBA has a high *in vivo* safety profile and has already been approved by US Food and Drug Administration for use in the treatment of urea-cycle disorders, thalassemia and cystic fibrosis (Collins et al. 1995; Rubenstein & Zeitlin 1998). Recently, PBA has also been shown to have beneficial effects on several mouse models of neurodegeneration. In a transgenic mouse model of Alzheimer's diseases that expresses the human mutant isoform of amyloid precursor protein PBA reversed spatial learning and memory deficits (Ricobaraza et al. 2009). PBA attenuates neuropathogenic effects in mouse models of Parkinson's disease induced by human α -synuclein A30P + A53T transgene (Ono et al. 2009). In another mouse model of Parkinson's disease induced by oral administration of rotenone, PBA significantly inhibits α -synuclein accumulation and aggregation (Inden et al. 2007).

Given that about 8% of the human genome is composed of sequences of human endogenous retroviruses (HERVs) (Lander et al. 2001; Griffiths 2001) and that these HERV gene sequences are able to express biologically active envelope proteins (Cheynet et al. 2005), it is not surprising that some of these HERV envelope proteins may have cellular functions. Unfortunately, under certain conditions, activation, overexpression or mutations in these *env* gene sequences could result in a spectrum of disease phenotypes, including neurodegeneration (Antony et al. 2004; Antony et al. 2007). In addition to HERVs noted above, a number of retroviruses including HIV and *ts1* have been shown to cause a spectrum of nervous system diseases (Gonzalez-Scarano 1995; Power 2001). In lieu of a designated mouse model for HIV-associated dementia (HAD), the *ts1* mouse model of neurodegeneration has been considered a suitable surrogate (Clark et al. 2001; Gonzalez-Scarano 1995). Recent reports show that astrocyte infection is much more extensive than previously reported in human patients with HAD (Churchill et al. 2009) and that ER stress occurs in the CNS of HIV-positive individuals (Lindl et al. 2007). Thus, ER stress resulting from the oxidative stress may still be a possible cause of astrocyte damage and neuronal cell loss in the CNS resulting in HAD. The fact that other proteins such as β -amyloid accumulates in HAD brain cells (An & Scaravilli 1997; Esiri et al. 1998; Rempel & Pulliam 2005; Achim et al. 2009) provides a strong support for the notion that accumulation of β -amyloid and other proteins could be the result of oxidative stress and ER stress in HIV-infected CNS cells (Lindl et al. 2007). Interestingly, in neurons of *ts1* infected brainstem, we also observed the presence of "Lewy bodies", which are formed as a result of accumulation of α -synuclein, a pathologic hallmark of Parkinson's disease (Stoica et al. 2000). Since *ts1* does not infect neurons, the accumulation of these Lewy bodies is not due to virus infection but rather indirectly caused by *ts1*-mediated oxidative stress, which leads to accumulation of

α -synuclein in neurons. Interestingly, PBA has also been shown to attenuate the pathogenic potency of human α -synuclein accumulation in the transgenic mouse model of Parkinson disease (Ono et al. 2009).

In conclusion, the data presented in the current study on the pathogenic mechanism of the *ts1*-induced neurodegeneration and its prevention with PBA treatment may provide new insights into the utilization of PBA as a potential therapeutic molecule. PBA treatment is likely a beneficial intervention to prevent neurological diseases not only in human retrovirus associated neurodegeneration, but also in many neurodegenerative diseases that are associated with protein accumulation and/or aggregation and ER stress. 1.5

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Research Highlights

In this study, we assessed whether treatments that reduce the accumulation of precursor envelope protein gPr80^{env} of *ts1*, a mutant of Moloney murine leukemia virus (MoMuLV), in the ER of infected astrocytes provided a protective effect against ER stress and cell death. We show that treatment with phenylbutyric acid (PBA) can prevent the unfolded protein response (UPR), ER stress and cell death in cultured *ts1*-infected astrocytes. The protective effect of PBA is associated with its ability to reduce gPr80^{env} accumulation and to increase the expression of proteins involved in protein folding in the ER, such as protein disulfide isomerase (PDI) and ERp44, rather than by decrease mRNA levels of gPr80^{env} or alter the proteasomal degradation process for gPr80^{env}. In infected mice treated with PBA we also noted a reduction in the severity of the neuropathology in brainstem tissues and a delayed onset of paralysis. These results show that PBA is a potentially effective drug for the treatment of neurodegeneration caused by misfolded protein accumulation in cells of the CNS.

Abbreviation used

ER	endoplasmic reticulum
UPR	unfolded protein response
PBA	Phenylbutyric acid
MoMuLV	Moloney murine leukemia virus
PERK	PRK-like ER kinase
ATF6	activating transcription factor 6
IRE1	inositol-requiring kinase 1
eIF2α	eukaryotic initiation factor α
ATF4	activating transcription factor 4
CHOP	the transcription factor C/EBP homologous protein
PDI	protein disulfide isomerase

ERp44	ERp57 and ERp72, endoplasmic reticulum proteins 44, 57 and 72
DMEM	Dulbecco's modified Eagle's medium
PCA	Primary cultured astrocytes
GFAP	glial fibrillary acid protein
MOI	multiplicity of infection
FITC	fluorescein
dpi	days post-infection
H&E	hematoxylin and eosin
DAPI	4,6'-diamidino-2-phenylindole
HERV	human endogenous retroviruses
HAD	HIV-associated dementia

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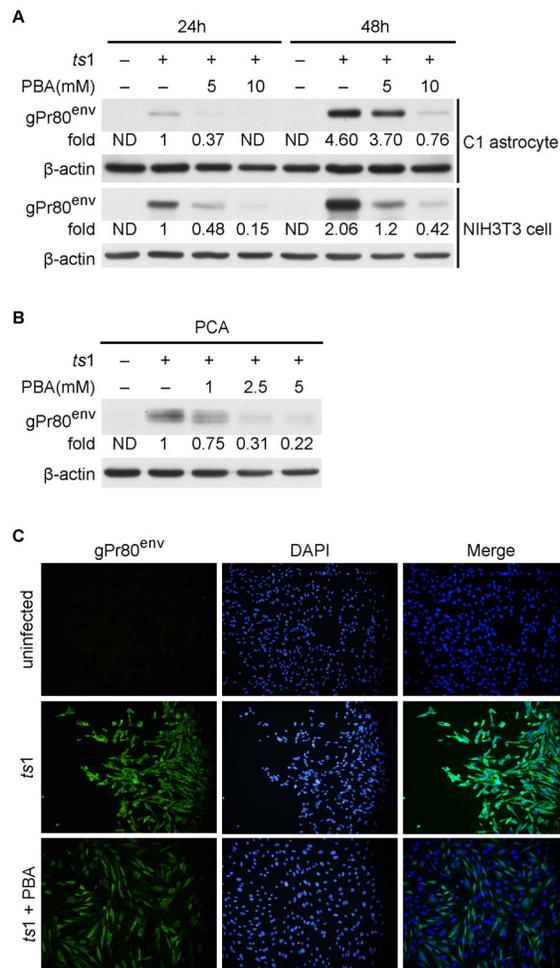


Figure 1. PBA decreases accumulation of gPr80^{env} in *ts1*-infected C1 astrocytes, primary astrocytes and NIH3T3 cells

A. C1 astrocytes and NIH3T3 cells were infected with the *ts1* virus at a MOI of 10, and PBA was added to the indicated cultures for 24 or 48 h after infection. Levels of gPr80^{env} in the whole cell lysates were compared using Western blot analysis. ND: not detectable. **B.** PCAs were treated with PBA for 96 hours, and cell lysates were then subjected to Western blot analysis to compare their levels of gPr80^{env}. **C.** C1 cells were infected with *ts1* and treated with PBA as described above for 48 hours, and were then processed for immunofluorescent staining with gPr80^{env} antibody.

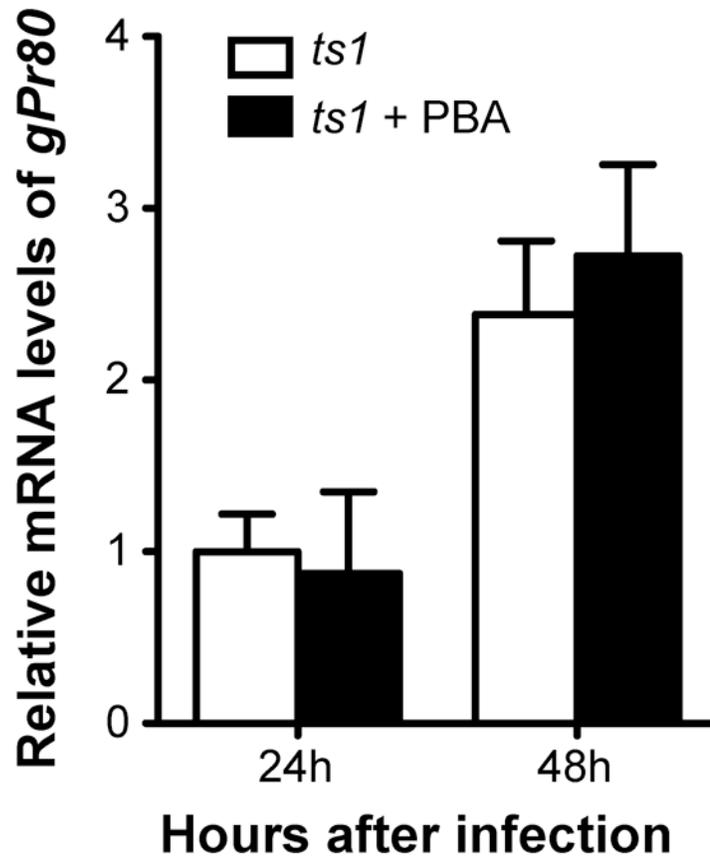


Figure 2. PBA treatment does not alter gPr80^{env} mRNA levels in infected C1 astrocytes
C1 astrocytes were infected with *ts1* at a MOI of 10, and then PBA was added at a concentration of 10 mM. After 24 or 48 h, RNA was isolated and the gPr80^{env} mRNA levels were quantitated by RT-PCR. $p < 0.05$ vs. control was considered statistically significant. Error bars represent mean \pm SEM from three independent experiments.

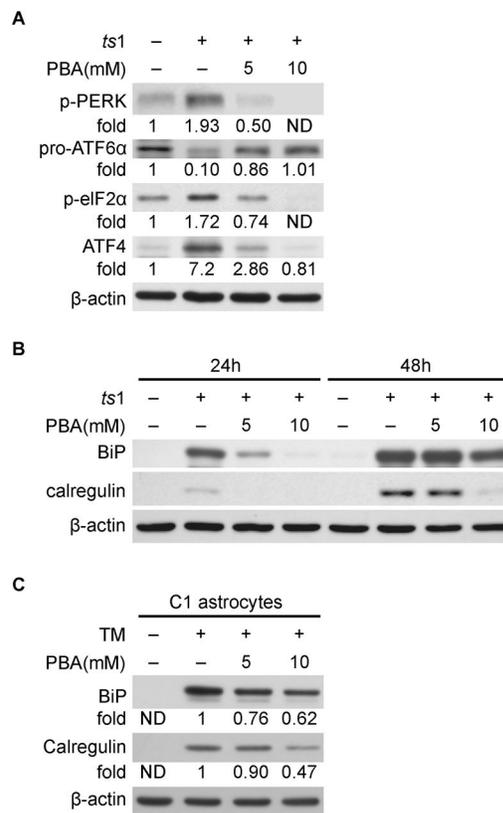


Figure 3. PBA reduces the UPR and ER stress in *ts1*-infected C1 astrocytes

A. C1 astrocytes were treated as described above for 48 hours, and whole cell lysates were then subjected to Western blot analysis to compare their levels of p-PERK, ATF6, p-eIF2 α and ATF4. **B.** C1 astrocytes were treated as described above. Levels of BiP and calregulin in cell lysates were compared by Western blotting. ND: not detectable. **C.** C1 astrocytes were treated with 5 μ g/ml tunicamycin (TM) with or without 5 or 10 mM PBA treatment for 24 hours. Levels of BiP and calregulin in whole cell lysates were compared by Western blotting. ND: not detectable.

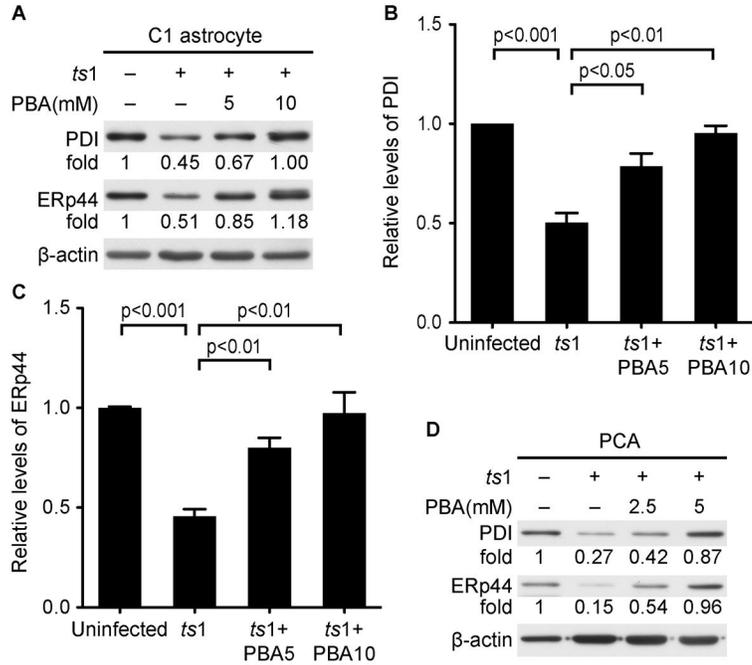


Figure 4. PBA enhances the expression of ER folding proteins in *ts1*-infected cells
A. C1 astrocytes were infected with *ts1* at a MOI of 10, and then PBA was added at a concentration of 5 or 10 mM for infected C1 cells. At 48 hours post infection, whole cell lysates were subjected to Western blotting to compare the expression of PDI and ERp44. Relative protein levels of PDI (**B**) and ERp44 (**C**) from three independent experiments described above were presented. Kodak Image Station was used to obtain intensity of band from Western blotting. PBA5: PBA at a concentration of 5 mM; PBA10: PBA at a concentration of 10 mM; **D.** PCAs were infected with *ts1* at a MOI of 10, and then PBA was added at a concentration of 2 or 4 mM for 96 hours. The protein levels of PDI and ERp44 were compared by Western blotting.

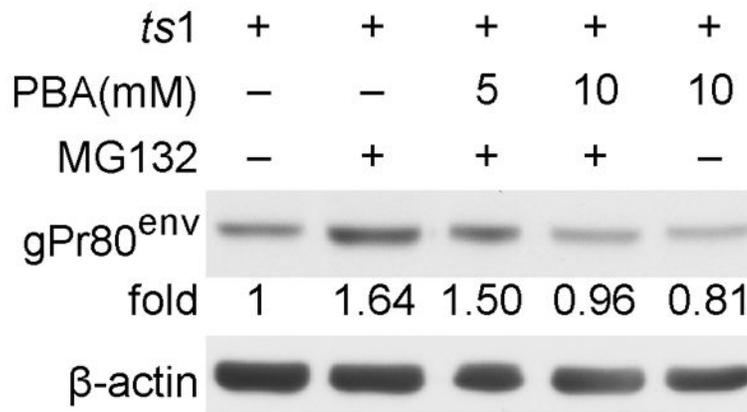


Figure 5. PBA treatment does not affect proteasomal degradation for gPr80^{env} in *ts1*-infected astrocytes

C1 cells were infected with *ts1* at a MOI of 10 for 24 hours, and then were treated with 2.5 μ M of MG132, either alone or together with 5 or 10 mM PBA. After 16 hours, the whole cell lysates were immunoblotted with gPr80^{env} antibody.

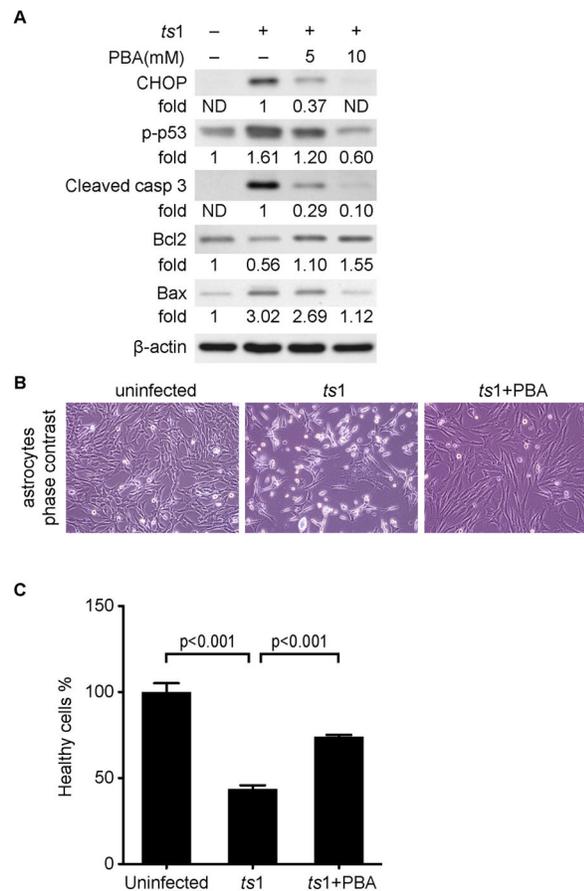


Figure 6. PBA prevents apoptosis in *ts1*-infected astrocytes, and also protects primary neurons exposed to spent medium from these cells

A. C1 astrocytes were treated as described above for 48 hours. Levels of the apoptotic pathway-related proteins CHOP, p-p53, cleaved caspase 3, Bcl-2 and Bax from whole cell lysates were analyzed by Western blotting. ND: not detectable. **B.** Phase contrast photomicroscopy shows that PBA protects *ts1*-infected astrocytes. C1 astrocytes were infected with *ts1* at a MOI of 10, and PBA was added at a concentration of 10 mM. Images were taken at 48 hours post infection. **C.** Cell viability assay with or without PBA treatment. C1 astrocytes were infected and treated as described above for 48 hours. Cells were stained firstly with propidium iodide and then with Hoechst 33342 as described in “Material and methods”. Cell viabilities from different treatments were compared using a healthy cell count excluding cells with propidium iodide staining. An average healthy cell number from uninfected control group was considered as to be 100%. The healthy cell number counted from other groups were averaged and expressed as healthy cell % \pm SEM relative to that of uninfected control group from three individual experiments.

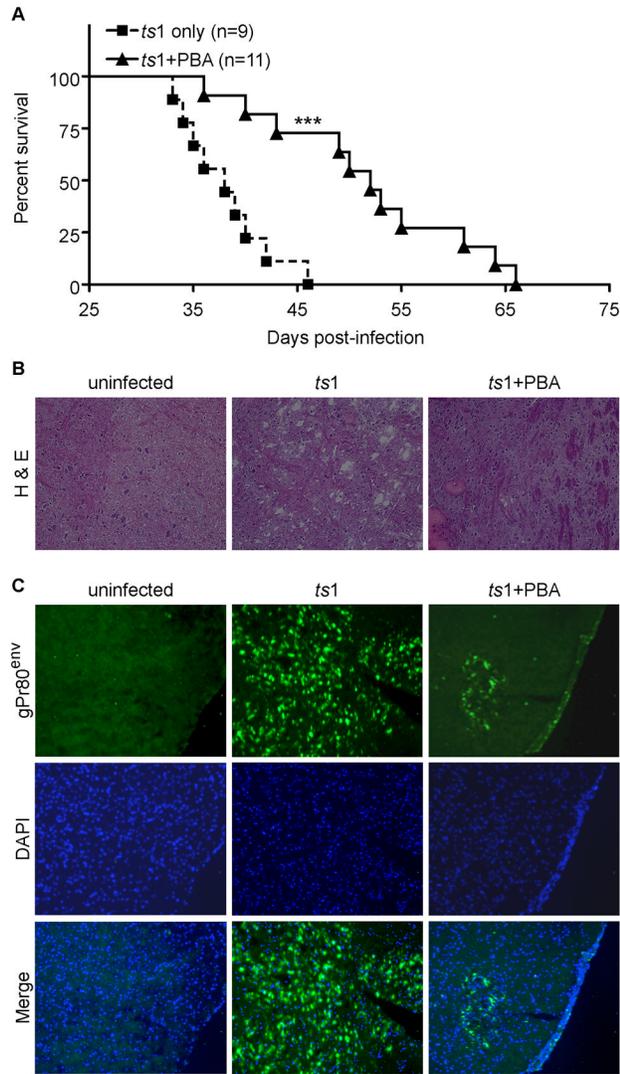


Figure 7. PBA delays paralysis and spongiform lesion formation in *ts1*-infected mice
A) Survival curves for PBA-treated *ts1*-infected mice (*ts1*+PBA, n=11) against survival curves for untreated *ts1*-infected mice (*ts1* only, n=9). ****p*<0.001. **B).** Frozen sections of brainstem from uninfected, *ts1*-infected untreated and PBA-treated mice were stained with H&E. Spongiform lesions are apparent in brainstems of *ts1*-infected untreated mice, while brainstems from PBA-treated mice showed much less vacuolation. **C).** Strong gPr80^{env} immunoreactivity is evident in *ts1*-infected untreated mice brainstems at 30 dpi, while brainstems from PBA-treated infected mice show considerably less gPr80^{env} at this time point.