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Endoplasmic Reticulum Protein BI-1 Modulates Unfolded Protein Response Signaling and Protects Against Stroke and Traumatic Brain Injury

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Abstract

Bax-Inhibitor-1 (BI-1) is an evolutionarily conserved cytoprotective protein that resides in membranes of the endoplasmic reticulum (ER). BI-1's cytoprotective activity is manifested in the context of ER stress, with previous studies showing that BI-1 modulates several ER-associated functions, including Unfolded Protein Response (UPR) signaling. Here we investigated the role of BI-1 in neuroprotection by generating transgenic mice in which BI-1 was constitutively expressed from a neuronal-specific promoter. Cultured primary cortical neurons from BI-1 transgenic mouse embryos exhibited greater resistance to cell death induced by agents known to cause ER stress compared to their non-transgenic counterparts. While brain morphology and vasculature of BI-1 mice appeared to be unchanged from normal non-transgenic mice, BI-1 transgenic mice showed reduced brain/lesion volumes and better performance in motoric tests, compared with non-transgenic littermates, in two models of acute brain injury – stroke caused by middle cerebral artery occlusion (MCAO) and traumatic brain injury (TBI) caused by controlled cortical impact. Furthermore, brain tissue from BI-1 transgenic mice showed reduced levels of apoptotic cells and reduced induction of markers of ER stress after brain injury, including CHOP protein expression. In summary, our findings demonstrate that enforced neuronal expression of BI-1 reduces ER stress and provides protection from acute brain injury, suggesting that strategies for enhancing BI-1 expression or activity should be considered for development of new therapies for counteracting the consequences of stroke and acute brain trauma.

Keywords

Neuroprotection; Unfolded Protein Response; Brain Injury

1. INTRODUCTION

Neuronal cell death in the context of neurodegeneration, stroke, and traumatic brain injury (TBI) constitutes a leading cause of morbidity and mortality, with few highly effective therapeutic options available. Various studies have suggested a role for endoplasmic reticulum (ER) stress in neuronal cell death (Bredesen et al., 2006; Kim et al., 2008). Multiple disturbances can cause accumulation of unfolded proteins in the ER, triggering an

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evolutionarily conserved response termed the “unfolded protein response” (UPR) (Frand et al., 2000; Uehara et al., 2006).

Among the UPR signaling pathways most clearly linked to induction of cell death is IRE1 (Kim et al., 2008). IRE1α is a type I transmembrane protein, which contains both a Ser/Thr-kinase domain and an endoribonuclease domain, the latter which processes an intron from X box-binding protein-1 (XBP-1) mRNA, rendering it competent for translation to produce the 41 kDa XBP-1 protein, a bZIP-family transcription factor. XBP-1 binds to promoters of several genes involved in UPR, and thus seems to be generally protective. In contrast, the protein kinase activity of IRE1α has been linked to cell death induction. Though IRE1’s substrates are not entirely defined, it shares in common with many members of the Tumor Necrosis Factor (TNF) receptor family the ability to bind adapter protein TRAF2 (Habelhah et al., 2004). IRE1 activates downstream protein kinases previously implicated in immunity, inflammation, and apoptosis, particularly Apoptotic Signaling Kinase-1 (Ask1), which causes Jun-N-terminal kinase (JNK) activation (Nishitoh et al., 2002), an event linked to post-translational modifications in several apoptosis proteins (Lei and Davis, 2003; Putcha et al., 2003; Srivastava et al., 1998; Wei et al., 2008). IRE1 also causes downstream activation of p38 MAPK, which phosphorylates and activates transcription factor CHOP, a transcriptional regulator induced by ER stress that is known to control expression of various apoptosis genes (Lei and Davis, 2003; McCullough et al., 2001; Putcha et al., 2003; Puthalakath et al., 2007; Wang and Ron, 1996; Zou et al., 2008). In addition, IRE1 is a focal point for the ER-targeted activities of some Bcl-2-family proteins, with pro-apoptotic Bax and Bak proteins interacting with and activating IRE1 (Hetz et al., 2006).

By functional screening of cDNA libraries for inhibitors of yeast cell death induced by ectopic expression of mammalian Bax, we discovered BI-1, Bax Inhibitor-1 (Xu et al., 2000). The cytoprotective activity of BI-1 is intimately linked to ER stress. The evolutionarily conserved BI-1 protein has several transmembrane domains, and resides in ER membranes (Chae et al., 2003; Chae et al., 2004). Over-expression of BI-1 protects against both caspase-dependent and caspase-independent cell death stimulated by agents that induce ER stress (Chae et al., 2004). The proteins with which BI-1 interacts in ER membranes include anti-apoptotic Bcl-2 family proteins (Bcl-2; Bcl-X_L) and IRE1 (Lisbona et al., 2009; Xu and Reed, 1998). In this report, we generated transgenic mice with enforced expression of BI-1 in neurons to explore the *in vivo* role of BI-1 and ER stress in acute brain injury.

2. RESULTS

2.1. Generation of transgenic mice with enforced BI-1 expression in neurons

Expression of the endogenous *BI-1* gene varies in response to cellular stress (Bailly-Maitre et al. 2006; Blais et al., 2004). To enforce continuous expression of BI-1 in neurons, we created an expression construct using the neuron-specific enolase (NSE) promoter for driving BI-1 transgene expression (Figure 1A), similar to prior studies of other types of cytoprotective genes (Kermer et al., 2003). Because human and mouse BI-1 proteins are highly similar (98% amino-acid similarity; 93% identity) and show a high degree of functional equivalence, we utilized a cDNA encoding human BI-1 with a C-terminal Hemagglutinin (HA) epitope tag for convenient immunodetection. Several founder mice were bred to establish lines, which were compared with respect to transgene-derived BI-1 mRNA levels in brain tissue (Figure 1B), revealing 3 transgenic (TG) lines with robust transgene expression. The production of BI-1-HA protein in brain tissue was confirmed by immunoblotting, whereas no expression was detected in other tissues such as spleen, kidney, heart, and liver (Figure 1C and data not shown), confirming tissue specificity. Immunohistochemical analysis using anti-HA antibody confirmed neuronal expression of

BI-1 in transgenic mice, showing broad expression in nearly all types of neurons (supplemental data). A complete histological analysis of brains from BI-1 transgenic mice was also performed. Overall, the brain architecture was normal for mice expressing BI-1 with proper development of all brain regions. In addition, no anatomical differences in the brain vasculature were observed, in particular the Circle of Willis, comparing wild-type with transgenic animals.

2.2. BI-1 expressing cultured neurons show resistance to cell death

Prior to initiating *in vivo* studies of brain injury, we investigated the effects of BI-1 transgene expression using cultured embryonic neurons from our TG mice, making side-by-side comparisons with non-transgenic (wild-type; WT) embryos from the same pregnant mother. BI-1 transgene expression significantly protected against cell death induced by L-glutamate (which binds NMDA receptors and causes excessive Ca^{2+} entry and induces nitric oxide production [excitotoxicity]), thapsigargin (inhibitor of ER Ca^{2+} ATPase, which causes release of ER Ca^{2+} into cytosol and induces ER stress due to defects in Ca^{2+}-dependent ER chaperones such as Calnexin), and hypoxia (which alters redox balance in the ER and causes protein misfolding due to defects in disulfide bond formation) (Figure 2).

Using cultured neurons from BI-1 and WT embryos, we also evaluated expression of CHOP protein, an indicator of ER stress. All 3 UPR pathways (IRE1, PERK, ATF6) make contributions to induction of CHOP expression during ER stress (reviewed in (Ron and Walter, 2007; Xu et al., 2005)). Immunoblot analysis of lysates from WT and TG cultured neurons showed that L-glutamate induced increases in CHOP protein levels in WT but not BI-1 expressing neurons (supplemental data). Thus, BI-1 transgene expression modulates ER stress signaling in neurons.

2.3. Enforced expression of BI-1 protects against stroke and inhibits induction of ER stress during brain ischemia-reperfusion

Previously, we observed that *BI-1* gene knock-out in mice results in increased sensitivity to stroke, using a middle cerebral artery occlusion (MCAO) model (Chae et al., 2004). Using the same MCAO model, we compared WT and TG mice with respect to functional and histopathological outcome. Mice were subjected to 2 h MCAO, followed by 2 h or 24 h reperfusion. Physiological parameters such as arterial blood pressure, arterial pO_2, arterial pH, and hematocrit measured in TG and non-TG mice were not significantly different prior to and after MCAO (not shown). Visual inspection of the anatomy of intracranial arteries (circle of Willis, MCA) did not reveal obvious differences between BI-1 TG and non-TG mice. Neurologic evaluation was performed at 2 h and 24 h post reperfusion. The neurological scores of the BI-1 TG mice showed significant improvement as indicated by a lower deficit score compared with WT control littermates at 24 h of reperfusion (1.0±0.3 for BI-1 TG; 2.7±0.3 for WT; *p=0.002) (Figure 3A).

Two methods of assessing stroke injury were utilized, employing serial coronal sections. First, stroke volume was determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining, showing that infarct volumes were significantly reduced in BI-1 expressing transgenic mice (17.7 ± 2.2 mm^3; $n = 10$) compared with age-matched WT controls (36.4 ± 3.7 mm^3; $n = 5$) measured at 24 h reperfusion ($p < 0.01$) (Figure 3B). Second, using a separate cohort of mice (n=6 WT; n=4 TG), serial coronal brain sections were prepared from sacrificed mice at 2 h and 24 h of reperfusion, fixed, and stained with Masson's trichrome to identify pyknotic degenerating neurons and to better visualize demarcation of infarct core and penumbra. Using digital image analysis, infarct areas were measured, and infarct volume was calculated showing a significant reduction in average infarct volume in BI-1 expressing mice compared to control littermates (*p=0.0006 at 2h) (Figure 3C). Thus, these data

indicate that the experimental stroke results in less histological and neurological damage in the BI-1 TG compared to control mice. Note the differences in the absolute areas of infarct lesions measured by TTC and Masson's trichrome are probably due to the differences in the sensitivity of the two methods, but that reduced lesion volumes were observed in BI-1 transgenic mice with both techniques.

Brain tissue from mice subjected to MCAO was analyzed by immunoblotting for markers of ER stress. For these studies, we measured protein markers early, immediately following 2 h of ischemia, before extensive tissue destruction occurred. The brains of BI-1 trangenic mice subjected to ischemia-reperfusion insult show reductions in several biochemical markers of ER stress, implying that BI-1 blunts ER stress signaling *in vivo* (Figure 3D). Among the ER stress events assayed, clear reductions were observed in expression of CHOP and Grp78 proteins. Modest but statistically significant reductions in phosphorylation of c-JUN were also detected in BI-1 transgenic mice subjected to stroke injury.

We used quantitative immunohistochemical analysis to further evaluate the effects of BI-1 on CHOP expression in the setting of stroke. Again, measurements were made early, immediately following 2 h of ischemia, before extensive tissue destruction. The prevalence of CHOP immunostaining was significantly reduced in the infarct region (Figure 3E; *p<. 0001) and in the entire ipsilateral hemispheres (not shown) of BI-1 transgenic compared to non-transgenic mice.

2.4 BI-1 protects in a model of traumatic brain injury (TBI) and inhibits signaling events associated with ER stress

In addition to the stroke model, we employed a controlled cortical impact (CCI) model (You et al., 2008) of experimental brain trauma to further explore BI-1 role in neuronal survival. To investigate the effect of enforced expression of BI-1 in neurons on post-traumatic functional performance, the wire grip test was used to assess motor function. BI-1 TG mice performed significantly better than the control cohort ($p<0.001$) (Figure 4A). To compare histopathological outcomes after brain trauma in both experimental groups, the lesion volumes were measured from Masson's trichrome-stained sections. In brain-injured BI-1 TG mice, the average lesion volume was significantly reduced at 2 h, 6 h, 24 h and 2 weeks after CCI compared to the control animals (Figure 4B; *p=0.02; **p=0.002; ***p=0.008; n=6 mice/time point/experimental group).

To determine whether enforced neuronal expression of BI-1 plays a protective role in CCI-induced neuronal cell death, the numbers of apoptotic neurons were assessed by TUNEL assay in cerebral ipsilateral hemispheres. Neuronal cell death was decreased in brains of BI-1 TG mice at 6 h and 24 h after brain injury (Figure 4C). No significant differences were observed in WT versus TG mice in contralateral hemispheres (not shown). The impact of BI-1 on expression of ER stress markers in the TBI model was also assessed. Induction of CHOP and phosphorylation of JNK and c-Jun were reduced in ipsilateral cerebral hemispheres of BI-1 mice compared to WT littermates (Figure 5; A: *p=0.0009, **p=0.02; ***p=0.05; B: *p=0.0008; C: *p=0.02, **p=0.006, ***p=0.04), showing that BI-1 over-expression inhibits induction of these markers of ER stress *in vivo* in the TBI model. Representative images of histological analysis are presented for TUNEL staining and for phospho-c-JUN and CHOP immunohistochemistry in Figure 6. Thus, BI-1 over-expression in brain also protects in a model of TBI, reducing markers of ER stress *in vivo*.

3. DISCUSSION

When unfolded proteins accumulate in the ER, resident chaperones become occupied with protein folding, releasing transmembrane ER signaling proteins responsible for the UPR.

Among the critical transmembrane ER signaling proteins are PERK, IRE1, and ATF6. Together, these proteins induce signal transduction events that ameliorate the accumulation of misfolded proteins in the ER (reviewed in Rao and Bredesen, 2004; Schroder and Kaufman, 2005; Shen et al., 2004). When excessive or prolonged, however, ER stress can result in cell death. Contexts in which ER stress-induced cell death has been implicated include cerebral ischemia-reperfusion injury and protein aggregation disorders, including most neurodegenerative diseases and several inherited diseases where mutant proteins with folding problems are produced (reviewed in Bredesen et al., 2006; Kim et al., 2008; Xu et al., 2005).

By generating transgenic mice with enforced expression of BI-1 in neurons, we show a protective role of this ER membrane protein in two models of acute brain injury. Because hypoxia has been implicated as a modulator of *bi-1* gene expression (Bailly-Maitre et al., 2006; Blais et al., 2004), we used a constitutive promoter (NSE) to enforce expression of BI-1 protein in neurons of transgenic mice. Previously, we demonstrated a possible role for BI-1 in intrinsic protection from cerebral ischemia by studies of gene knock-out mice, which suffered larger infarcts following transient middle cerebral artery occlusion (Chae et al., 2004). Thus, the findings reported here provide further evidence that BI-1 is an important regulator of neuronal survival *in vivo* during acute stress and injury. Long-term functional outcome studies should be conducted to further evaluate the role of BI-1 in recovery from stroke injury. Also, it should be recognized that animal models of stroke and TBI have historically not been predictive of outcome in human clinical trials, and thus results must be interpreted with caution.

As a regulator of ER-initiated cell death and cell stress signaling, the observations made with BI-1 transgenic mice provide further evidence of an important role for ER-driven pathways in neuronal injury. In this regard, reduced blood flow resulting from arterial occlusion, hypotension, and trauma results in tissue hypoxia and hypoglycemia, which cause protein misfolding and ER stress. Reperfusion of the affected tissues then triggers oxidative stress, with production of Nitric Oxide (NO), a mediator of protein nitrosylation, and other reactive oxygen species (ROS) that alter cellular redox-dependent reactions, interfere with protein disulfide bonding and result in protein misfolding (Gotoh and Mori, 2006). Dysregulation of ER Ca^{2+} homeostasis may also contribute to the neuropathological consequences of acute brain injury (Kim et al., 2008).

The mechanisms by which BI-1 affords neuroprotection *in vivo* may be multiple. First, in other studies, BI-1 has been reported to interact with IRE-1 in ER membranes, interfere with Bax/Bak binding to IRE-1, and inhibit IRE-1 signaling (Bailly-Maitre et al., 2010; Lisbona et al., 2009). Among the signaling events initiated by IRE1 activation is stimulation of stress kinases downstream of Ask1, which include JNK and p38MAPK. These stress kinases are known to phosphorylate various members of the Bcl-2 family, promoting apoptosis (Kim et al., 2008). In BI-1 transgenic mice, reductions in phosphorylation of the JNK substrate c-JUN were observed in brain tissue for both the MCAO (stroke) and TBI models, consistent with the notion that BI-1 suppresses IRE-1 signaling. Of course, many stimuli besides IRE-1 could lead to JNK activation and c-Jun phosphorylation in the context of acute brain injury, and thus this observation by itself does not causally link BI-1 to IRE-1 pathway suppression. Also, some of BI-1's impact on UPR signaling events could be secondary consequences of other protective mechanisms that reduce ER stress (such as antioxidant defense responses, *see below*), as suggested by the observation that enforced expression of BI-1 also blunted IRE-1-independent UPR signaling events such as phosphorylation of eIF2alpha and induction of Grp78. Interestingly, of the 3 major initiating UPR signaling proteins (IRE1, PERK, ATF6), only IRE1 is conserved in yeast, suggesting a possible explanation for why BI-1 shows protection against Bax-induced killing in yeast.

Second, UPR signaling induces expression of CHOP, with all three major UPR pathways (IRE-1, PERK, ATF6) making contributions to the transcriptional induction of this gene that encodes a C/EBP-family bZIP transcription factor induced by ER stress (Oyadomari and Mori, 2004; Ron and Walter, 2007; Xu et al., 2005). *Chop*−/− mice reportedly suffer less tissue loss after stroke injury, implying a causal role for this mediator of ER stress in neuronal cell death *in vivo* (Tajiri et al., 2004). Nitric Oxide (NO), a known mediator of brain injury during stroke, induces *Chop* gene expression in cultured rodent neurons, and mice lacking the gene encoding inducible NOS (iNOS) have reduced *Chop* induction and display decreased sensitivity to brain ischemia (Iadecola et al., 1997). The CHOP transcription factor modulates the expression of several apoptosis-regulatory genes, including suppressing the gene encoding Bcl-2 (anti-apoptotic) and inducing the genes encoding Bim and DR5/TRAIL Receptor (pro-apoptotic) (Puthalakath et al., 2007). We observed reduced CHOP protein expression in BI-1 transgenic mouse brains following MCAO (stroke) and TBI, as well as less CHOP production in cultured neurons from BI-1 transgenic mice. Thus, reductions in CHOP protein levels may account for at least some of the neuroprotective phenotype observed when BI-1 expression is enforced in neurons.

Third, though not examined here, modulation of the Nrf2/Keap1 pathway is another action of BI-1 that has been reported in the literature, at least in cultured cells. Nrf/Maf-family transcription factors induce the expression of diverse genes that contain conserved cis-acting elements (Anti-Oxidant Response Elements [AREs]) and that encoding proteins with antioxidant activity, including heme oxidase-1 (HO-1) (Kang et al., 2005). The transcription factor Nrf2 is sequestered in the cytosol by Keap1, a redox-sensitive protein, which releases Nrf2 upon modification of regulatory cysteines in Keap1. BI-1 over-expression activates the Nrf2 pathway via an undefined mechanism in cultured cells (Lee et al., 2007), thus representing an additional potential neuroprotective mechanism invoked by BI-1.

Finally, BI-1 also regulates ER Ca^{2+} homeostasis. Cells over-expressing BI-1 have reduced luminal concentrations of Ca^{2+} in the ER, whereas cells in which BI-1 expression has been reduced (shRNA) or ablated (by homologous gene recombination) have higher resting levels of free Ca^{2+} in the ER (Chae et al., 2004; Westphalen et al., 2005; Xu et al., 2008). The mechanism by which BI-1 regulates ER Ca^{2+} is unknown, but it correlates with an increase in passive Ca^{2+} efflux from the ER. The effect of BI-1 on ER Ca^{2+} phenocopies Bcl-2 and Bcl-X_L, which are also known to integrate into ER membranes and to regulate ER Ca^{2+}. It has been reported that these anti-apoptotic proteins regulate ER Ca^{2+} through effects on Inositol Tri-phosphate Receptors (IP3Rs) (Rong et al., 2008). Interestingly, gene ablation studies by our laboratory have shown that BI-1 is required for ER Ca^{2+} regulation by Bcl-X_L (Xu et al., 2008), suggesting that BI-1 collaborates with Bcl-2/Bcl-X_L to regulate ER Ca^{2+} homeostasis. Thus, modulating internal Ca^{2+} stores is another potential mechanism by which BI-1 may contribute to neuroprotection.

When taken together with previous reports, our findings with BI-1 transgenic mice demonstrate an important role for BI-1 in conferring neuronal resistance to cellular stress. This resistant state is manifested at minimum in the context of acute injury states that involve ischemia-reperfusion (MCAO/stroke; and TBI), and is accompanied by modulation of UPR signaling events associated with ER stress. Accordingly, we propose that therapeutic strategies should be considered for bolstering BI-1 expression or BI-1 activity in neurons.

4. EXPERIMENTAL PROCEDURE

4.1. Plasmids

A plasmid was constructed for expression of HA-tagged human BI-1 (hBI-1 HA) under the control of the neuron-specific enolase (NSE) promoter. A cDNA encoding human BI-1 with

C-terminal HA tag (Xu and Reed, 1998) was amplified by PCR from pcDNA3 BI-1-HA with Nhe I site engineered at the 5′ end and XbaI site at 3′ end. The PCR product was then digested by Nhe I and Xba I and cloned into corresponding NheI and XbaI sites of pNSE-Luc (Kermer et al., 2003). After amplification of the plasmid, the NSE-BI-HA transcription unit was excised by restriction digestion and gel-purified.

4.2. Generation of BI-1 transgenic mice

After purification and dialysis, the linealized DNA was micro-injected into mouse zygotes (from FVB/N mice) prior to pronuclei fusion. After reaching the blastocyst stage *in vitro*, the embryos were transferred into pseudopregnant recipient mice. From the litters, 7 founder animals with independently integrated transgenes were identified. Three lines with the highest expression were further characterized. Transgenes were bred for at least 9 generations onto a C57BL/6 strain background. All animal work was approved by the Sanford-Burnham IACUC and conducted under guidelines of the NIH office of laboratory animal welfare (OLAW). The animal facility is accredited by AAALAC (A-3053-1). Genotyping was routinely performed by PCR analysis of genomic DNA using NSE primer (5′-CCACTCCTGCTCTCTCTG-3′) and BI-1 primer (5′-GGCTGACATCAAGATACCTCC-3′).

4.3. RT-PCR

Mice were sacrificed by cervical dislocation, and brains were dissected and flash-frozen in liquid nitrogen. The brains were ground thoroughly with a mortar and pestle in the presence of liquid nitrogen and decanted into an RNase-free tube. Total RNA was isolated with RNeasy maxi-kit (QIAGEN). The amount of RNA was quantified by measuring O.D.260/O.D.280 ratio with a spectrophotometer. The integrity of the RNA was verified by fractionating 5 μl of the RNA samples by agarose gel electrophoresis and staining with ethidium bromoide, revealing distinct 28S and 18S rRNA bands. Reverse transcription was performed with SuperScript™ II RNase H^- Reverse Transcriptase (Invitrogen) according to the manufacturer's instruction. Briefly, 1 μg RNA, 1 μl 500 ng/μl of oligo-dT anchor primer, 1 μl 10 mM dNTP, RNase-free H_2O (12 μl total volumn) were mixed and heated to 65 °C for 5 min, then chilled on ice. Next, 4 μl 5X First Strand Buffer, 2 μl 0.1M DTT, 1 μl RNase Inhibitor (Promega) and 1 μl Reverse Transcriptase were added and incubated at 42 °C for 50 min, followed by enzyme inactivation by heating at 70 °C for 15 min. PCR reactions were performed using Taq DNA polymerase from Qiagen with 2μl of the RT reaction product as template. The PCR conditions entailed heating at 94 °C for 2 min, followed by thermocycling at 94 °C for 15 sec, 55 °C for 30 sec, 72 °C for 1 min, for 30 cycles, followed by a final extension at 72 °C for 10 min. BI-1 primers used are specific for NSE-BI-1-HA transgene-derived cDNA, and included forward primer 5′ GACCGAGCAAAAGAGACTGG 3′ and reverse primer 5′AAGGCCAGGATCAACATGAG 3′. Control cyclophilin A primers were 5′ GCACTGGGCAGGTGTCC 3′ and 5′ GGGGTTGACAGCAATACAAA 3′. PCR products (10 μl) were analyzed by agarose gel-eletrophoresis with ethidium bromide staining.

4.4. Immunoblotting

Protein lysates were prepared, normalized for total protein content, and analyzed by SDS-PAGE/immunoblotting as described (Bailly-Maitre et al., 2006; Bailly-Maitre et al., 2010; Kermer et al., 2003).

4.5 Primary Neuron Culture and Cell Death Assay

Primary cultures of cerebrocortical neurons were obtained from embryonic brains at E15.5 according to published protocols (Ahlemeyer and Baumgart-Vogt, 2005). Approximately

175,000 cells were seeded onto coverslips coated with poly-D-lysine and laminin in 24-well plates and cultured for 3 weeks prior to treatments. Cell death stimuli included (a) exposure to 2–10 μM L-glutamate (in the presence of 10 μM glycine) for 20 minutes, followed by re-incubation in the original culture medium for 16–24 h; (b) exposure to hypoxia (0.2% O_2), hypoglycemia (culturing cells in serum-free medium with 1.8 mM $CaCl_2$) for 4–16 h, and (c) culturing with thapsigargin, or tunicamycin. To determine the percentage of apoptotic neurons, cells were fixed, then stained immunocytochemically with MAP2 (dendrites and perikarya stain, Sigma) and NeuN (nuclear stain; Chemicon MAB377) antibodies to visualize neurons, followed by counterstaining with DNA-binding fluorochrome DAPI. The percentage of apoptotic neurons (condensed nuclei) was determined by UV-microscopy, counting at least 100 cells/field in ten 20× fields for a total of 1,000 cells counted (n = 3 determinations).

4.6. Histology and Immunohistochemistry

Mice were anesthetized with isoflurane and transcardially perfused with phosphate-buffered saline (PBS) pH 7.4, followed by zinc-buffered formalin (Z-fix; Anatech, Inc., Battle Creek, MI). Excised brains were postfixed for 3–5 days, then 1.5 mm thick coronal slices were prepared and embedded into a single paraffin block so that all sections from each coronal slice could be stained on the same slide, ensuring identical staining conditions. In addition to H&E, brain sections were stained with Masson's trichrome (American Master*Tech Scientific, Inc.; Lodi, CA). Dewaxed tissue sections (4.0–5.0 μm) were immunostained as reported previously (Krajewska et al., 2009) using rabbit polyclonal antibodies to phospho-c-Jun (Ser73) (Cell Signaling Technology, cat.#9164) and phospho-SAPK/JNK (Thr183/Tyr185) (Cell Signaling Technology cat#9251), as well as mouse monoclonal antibody to GADD 153 (CHOP) (Santa Cruz ca#sc-7351). Application of the primary antibody was followed by incubation with goat anti-mouse or goat anti-rabbit polymer-based EnVision-HRP-enzyme conjugate (DakoCytomation). DAB (DakoCytomation) and SG-Vector (Vector Lab, Inc.; Burlingame, CA, USA) chromogens were applied, yielding brown and black colors, respectively.

Quantitative analysis was performed as described previously (Krajewska et al., 2009). Briefly, all slides were scanned at an absolute magnification of 400× [(resolution of 0.25 μm/pixel (100,000 pix/in.)] using the Aperio ScanScope CS system (Aperio Technologies; Vista, CA). The acquired digital images representing whole tissue sections were analyzed applying the Spectrum Analysis algorithm package and ImageScope analysis software (version 9; Aperio Technologies, Inc.) to quantify stainings using color deconvolution algorithms (Ruifrok and Johnston, 2001). Colorimetric intensity measurements were applied to Masson's trichrome- and antibody-stained tissues, using the nuclear algorithm.

4.7. Terminal Deoxyribonucleotidyl Transferase–mediated dUTP Nick End-Labeling (TUNEL) Assay

The detection of nuclei with fragmented DNA by TUNEL was accomplished using the ApopTag Peroxidase in situ Apoptosis Detection Kit (Chemicon) according to the manufacturer's instructions. Nuclear Red (DakoCytomation) was used as counterstain.

4.8. Immunofluorescence

Double labeling with chicken anti-neurofilament heavy chain polyclonal antibody (as a neuronal marker, CHEMICON, Cat. No. AB5539) and rat anti-HA antibody (to detect BI-1HA; Roche, Cat. No. 11 583 816 001) was performed on frozen brain sections, using previously established methods (Kadoya, et. al., 2008).

4.9. MCAO procedure

After an overnight fast, young adult male mice (~25–30 g; n=23 WT and 22 TG) were anesthetized and subjected to MCAO injury as described in detail previously (Kermer, et. al., 2003) using a silicone-coated 6-0 surgical nylon filament with a heat-rounded tip. The suture was advanced from the external carotid artery into the lumen of the internal carotid artery until it blocked the origin of the middle cerebral artery and rested at the base of the anterior cerebral artery. After a 2 h occlusion, perfusion was re-established by withdrawal of the suture until the tip cleared the lumen of the external carotid artery. Occlusion and reperfusion were verified by laser Doppler flowmetry to monitor relative cerebral blood flow. At 24 h of reperfusion, mice were subjected to neurologic evaluation briefly before sacrificing. Each animal was assigned a score of 0–4 according to the following scale: 0 – no observable neurological deficit; 1 – failure to extend the left forepaw; 2 – circling to the left; 3 – falling to the left; 4 – unable to walk spontaneously (Nakanishi et al., 2009). After completion of the experiment, animals were deeply anesthetized with isofluorane and transcardially perfused with 0.9% NaCl. Excised brains were dissected, cut into 1 mm slices and stained with 2% 2,3,5-triphenyltetrazoluidine (TTC). Infarct size was measured in a blind fashion using NIH 1.6 software, correcting values for edema as published (Kermer et al., 2003). Briefly, the area of infarction and the area of the ipsilateral hemisphere (mm^2) were calculated from TTC-stained slices, and the volumes (mm^3) were determined by integrating the appropriate area with the section interval thickness. The infarct volume was calculated using a formula (Swanson et al., 1990) in which infarct volume was calculated as a percentage of the contralateral hemisphere [100×(contralateral hemisphere volume − non-infarct ipsilateral hemisphere volume)/contralateral hemisphere volume].

4.10. Controlled Cortical Impact Model of Traumatic Brain Injury

WT and BI-1 TG mice (n=24 per experimental group (WT non-TG; BI-1 TG); 6 mice per time point (4 time points)) were anesthetized with 4% isoflurane in 70% N_2O and 30% O_2 and positioned in a stereotaxic frame. Using a head restraint, a 5-mm craniotomy was made using a portable drill and a trephine over the right parietotemporal cortex and the bone flap was removed (You et al., 2008). Mice were subjected to CCI using the benchmark stereotaxic impactor (Impact One™; myNeuroLab.com) with the actuator part mounted directly on a stereotaxic instrument. The impactor 3 mm tip accelerated down to the 1.0 mm distance, reaching the preset velocity of 3 m/s, and the applied electromagnetic force remained there for the dwell time of 85 ms, and then retracted automatically. The contact sensor indicated the exact point of contact for reproducible results. Afterwards, the scalp was closed with sutures, anesthesia discontinued, and mice allowed to recover.

4.11. Wire grip test

Vestibulomotor function was assessed using a wire-grip test (Bermpohl et al., 2006). Mice were placed on a metal wire (45 cm long) suspended 45 cm above a foam mat between 2 vertical bars. Mice were allowed to cross the wire for 60 sec. The latency that a mouse remained on the wire within a 60-sec interval was measured, and wire grip scores were quantified using a 5-point scale (Bermpohl et al., 2006; Hall et al., 1988). Mice that were unable to remain on the wire for less than 30 s were given a score of zero. A score of one point was given if the mouse failed to hold on to the wire with both sets of fore paws and hind paws together; two points were given if the mice held on to the wire with both fore paws and hind paws but not the tail; three points were given if the mouse used its tail along with both fore paws and both hind paws; four points were given if the mouse moved along the wire on all four paws plus tail; and five points were given if mice that scored four points also ambulated down one of the posts used to support the wire.

4.12. Assessment of lesion (TBI) and infarct (MCAO) volumes

Lesion volume was calculated as previously reported (Krajewska et al., 2009). Briefly, the Masson's trichrome–stained sections were digitalized using the Aperio scanning system. Using the pen tool, virtual slides were annotated by encircling the lesion/infarct edges (penumbra) between intact and pathologically changed brain tissue, and the lesion/infarct area (mm^2) was reported in the annotation window. The rostral–caudal dimensions of the foci were determined through the use of a stereotaxic atlas for the mouse brain (Paxinos et al., 2001), which was viewed electronically side by side with the coronal section images. Lesion/infarct volume was calculated by summing each lesion/infarction area multiplied by the distance between each coronal slice. Calculation are based on Cavalieri's method (Rosen and Harry, 1990) modified for the purpose of volumetric analysis performed on digital slides.

4.13. Statistical Analysis

Data were analyzed using the STATISTICA software package (StatSoft; Tulsa, OK). Statistical analyses of pairs of WT versus TG mice or specimins were performed using a one-way Student's *t*-test. A one-way analysis of variance (ANOVA) method was used to determine the significance of differences of multiple point data sets. *P* values <0.05 were reported as statistically significant.

RESEARCH HIGHLIGHTS

- BI-1 expressing cultured neurons show resistance to cell death.
- Enforced BI-1 expression inhibits ER stress and protects against stroke.
- BI-1 inhibits ER stress signaling in a model of traumatic brain injury (TBI).
- NSE-BI-1 TG mice have improved functional and histopathological outcome after TBI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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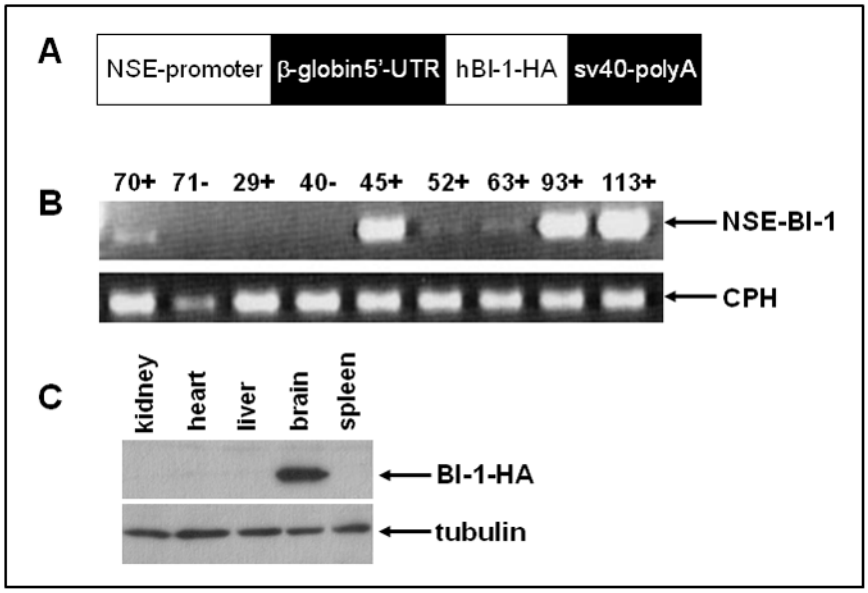

Figure 1. Production of transgenic mice over-expressing BI-1 in neurons

(a) The construct used to produce transgenic mice is depicted at top, consisting of neuron-specific enolase (NSE) promoter, a β-globin gene cassette with 5′ untranslated region (UTR) and intron, followed by cDNA encoding human BI-1 with C-terminal HA tag, followed by polyadenylation sequences (PolyA) from SV40 virus. *(b)* RNA samples from brains of offspring of various founder mice were analyzed by RT-PCR for expression of the transgene. Plus and minus signs correspond to transgene positive and negative mice (genotype), respectively. Note the high levels of BI-1 mRNA expression in mice #45, #93, and #113, which are the three founder lines selected for continued breeding. *(c)* Extracts from various tissues of a transgenic mouse were prepared, normalized for protein content, and analyzed by immunoblotting using anti-HA or γ–tubulin antibodies.

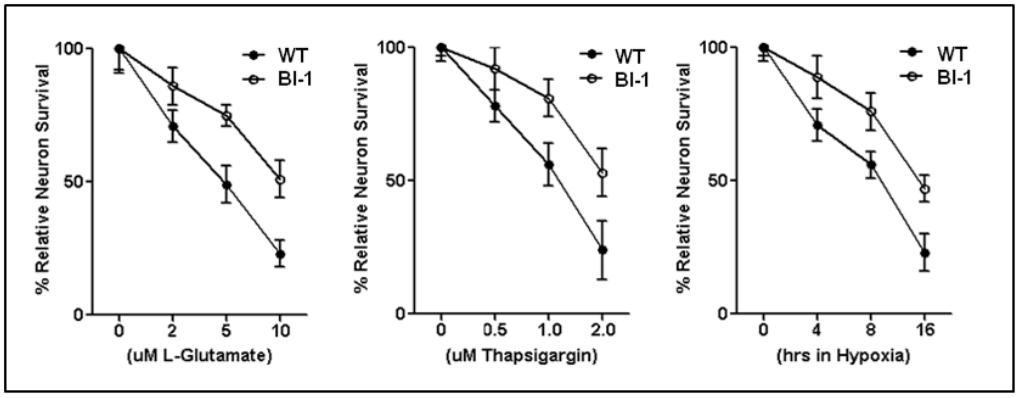

Figure 2. Transgenic expression of BI-1 in neurons of mice affords protection from agents known to cause ER stress

Neuron cultures were prepared from E15.5 day embryos of non-transgenic (WT, solid circles) or BI-1 transgenic (BI-1, open circles) mice and subjected to treatment with 2–10 μM L-glutamate for 20 minutes and allowed to recover in conditioned medium for 20 h (*left*) or cells were cultured with various concentrations of thapsigargin for 24 h (*middle*). Alternatively, cultured neurons were subjected to hypoxia (0.2% oxygen) for various times (*right*). Data represent mean ± SD (n = 4). Differences between WT and TG were statistically significant ($p<0.05$) as determined by ANOVA for all treatments.

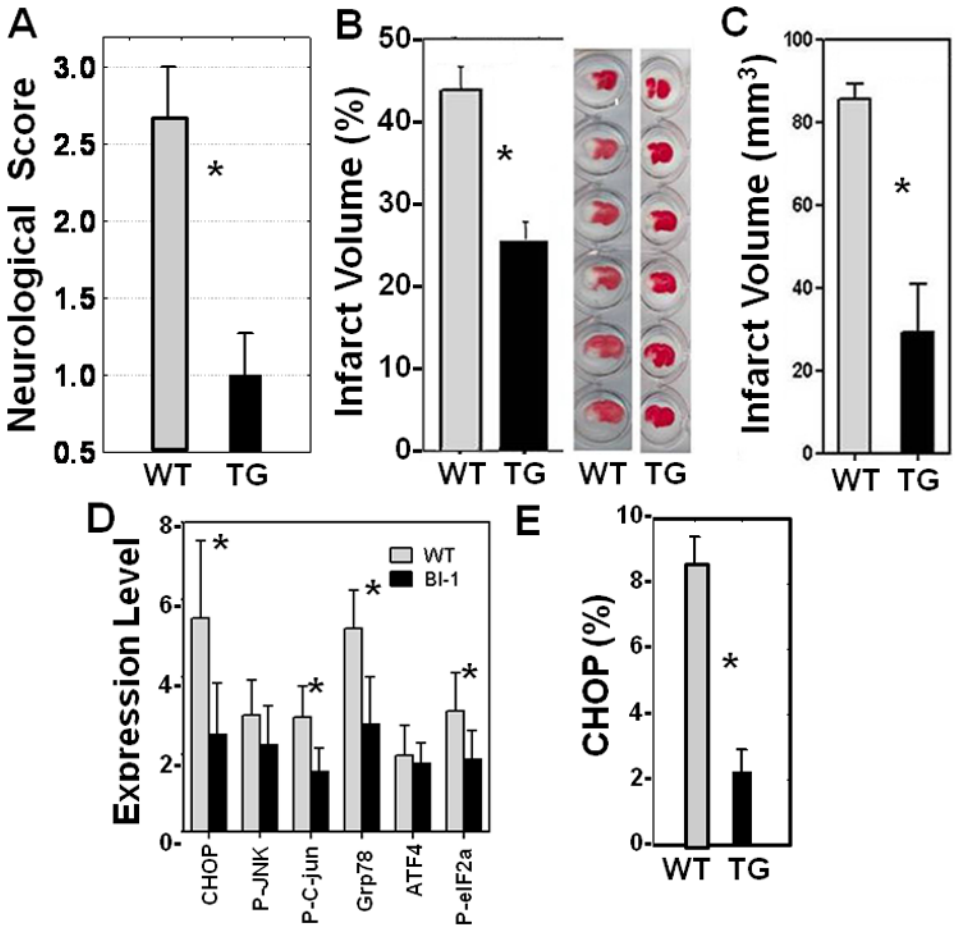

Figure 3. BI-1 reduces ER stress signaling and protects against stroke injury in mice

Groups of wild-type (WT) and BI-1 transgenic littermates at ~12 weeks of age were subjected to middle cerebral artery occlusion (MCAO) for 2 h, followed by 2 h or 24 h reperfusion. ***(A)*** The bar graph depicts average neurological scores (scale 0–4) for WT and TG mice at 24 h of reperfusion (*p=0.002). ***(B)*** Infarct volume corrected for edema (mean in nm^3±SEM) was measured in TTC-stained brain sections of wild-type (n=5) and BI-1 transgenic (n=10) mice sacrificed 24 h after 2 h MCAO (*p=0.007). ***(C)*** Serial coronal brain sections were stained with Masson's trichrome. Digitalized images were captured and the infarct volumes were measured from virtual images of brains from WT (n=6) and BI-1 TG (n=4) mice sacrificed at 2 h of reperfusion. Infarcts were annotated by encircling the lesion edges (penumbra) between intact and pathologically changed brain tissue, and the infarct area (mm^2) was reported in the annotation window. The rostral–caudal dimensions of the infarct were determined through the use of a stereotaxic atlas for the mouse brain. Distance (mm) between coronal coordinates of the slices determined the distribution of the infarct. Infarction volume was calculated by summing each infarction area multiplied by the distance between each coronal slice. Differences were confirmed to be statistically significant (*p=0.0006). ***(D)*** *H*omogenates of brain tissue from BI-1 TG (black bars) and WT littermate (grey bars) mice were prepared from the ipsilateral hemispheres of mice subjected to 2 h ischemia-injury (MCAO), normalized for protein content, and analyzed by immunoblotting using antibodies specific for various ER stress markers, including CHOP, ASK1, ATF4, Grp78, and phospho-JNK, phospho-c-JUN, phospho-eIF2a. The data for phosphorylated proteins was normalized for total levels of JNK, c-JUN, and eIF2a based on

immunoblotting with antibodies that recognize the proteins irrespective of phosphorylation (ratio phosphorylated/total protein). Data represent relative expression levels compared to the contralateral hemisphere (= 1.0) and are mean ± SEM (n = 3) (*$p < 0.05$). The experiment shown here represents biomarker analysis at 24 h of reperfusion. **(E)** The percentage of CHOP positive cells in the infarct and ipsilateral hemisphere was determined at 2 h of reperfusion by IHC and quantitative morphometry (mean±SEM; WT n=6, BI-1 TG n=4).

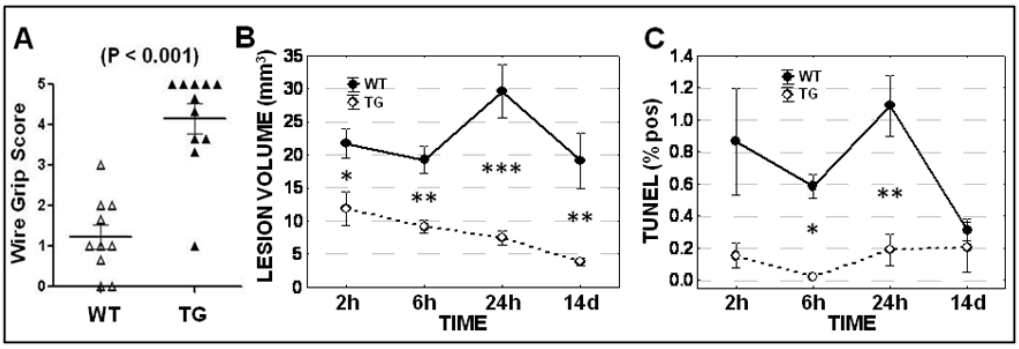

Figure 4. BI-1 provides protection in a model of traumatic brain injury

Mice were subjected to uniform controlled cortical impact injury. ***(A)*** Impairment in motor function was tested at 7 days post TBI by wire grip test. Scores were quantified using a 5-point scale (Hall et al., 1988) and compared between WT and TG mice (P<.001). Each symbol corresponds to an experimental animal. ***(B)*** Lesion volume was assessed at 24 h post injury, using the Aperio software to measure lesion area and Cavalier's method (mean ±SEM; n = 6 per time point per group) (Rosen and Harry, 1990). ***(C)*** The percentage of TUNEL-positive cells in the ipsilateral hemisphere was determined at 24 h post injury by quantitative morphometry (mean±SEM, n = 6 per group).

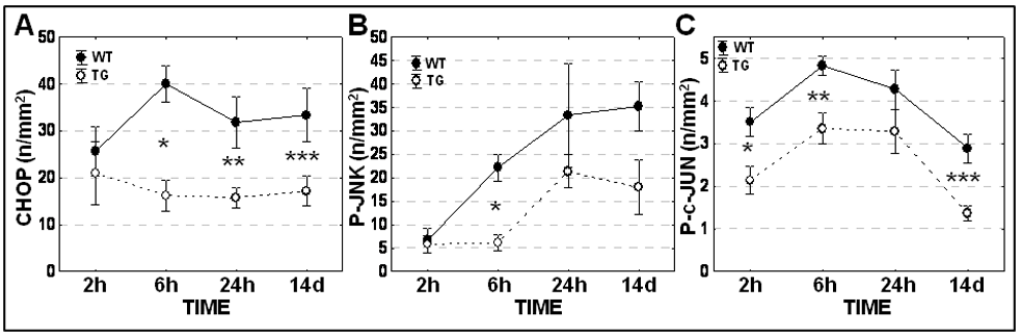

Figure 5. BI-1 inhibits induction of markers of ER stress in vivo in TBI model

WT (black circles) and BI-1 transgenic (white circles) mice were subjected to TBI, then mice (n = 6 per time point in each experimental group) were sacrificed and brain tissue was recovered at various times thereafter (2, 6, 24 h or 2 weeks), fixed, paraffin embedded, sectioned, and immunostained with antibodies specific for CHOP **(A)** or phospho-JNK **(B)**, and phospho-c-Jun **(C)**. Digitized images of ipsilateral hemispheres were annotated for morphometric analysis. Data represent number of positive cells/hemisphere area (mm^2) (mean±SEM).

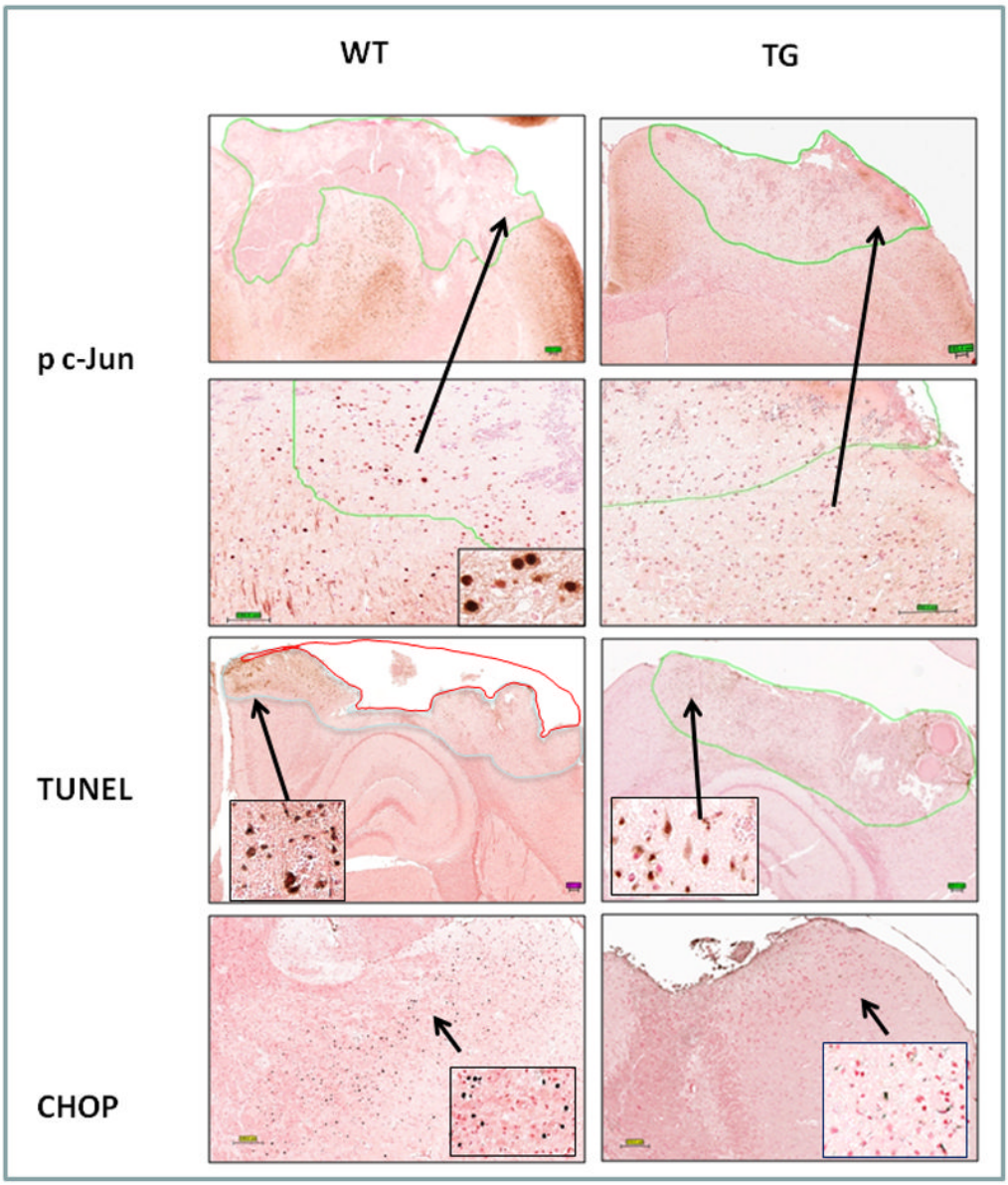

Figure 6. BI-1 reduces ER stress signaling and apoptosis after TBI in mice

Groups of wild-type (WT) and BI-1 transgenic littermates at ~12 weeks age were subjected to CCI brain injury. Serial coronal brain sections were prepared and stained for phospho-c-Jun, CHOP, and TUNEL and counterstained with Nuclear Red. Digitalized images were captured (Aperio scanning system) and lesion areas were annotated by encircling the lesion edges (penumbra) between intact and pathologically changed brain tissue. Nuclear algorithms were applied to quantify protein expression in the annotated lesion areas and ipsilateral hemispheres. Arrows placed on high magnification images point to the corresponding brain areas at low magnification. Bar = ~100 μm.