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Calcineurin β protects brain after injury by activating the unfolded protein response

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Abstract

The Ca²⁺-dependent phosphatase, calcineurin (CN) is thought to play a detrimental role in damaged neurons; however, its role in astrocytes is unclear. In cultured astrocytes, CN β expression increased after treatment with a sarco/endoplasmic reticulum Ca²⁺-ATPase inhibitor, thapsigargin, and with oxygen and glucose deprivation, an *in vitro* model of ischemia. Similarly, CN β was induced in astrocytes *in vivo* in two different mouse models of brain injury - photothrombotic stroke and traumatic brain injury (TBI). Immunoprecipitation and chemical activation dimerization methods pointed to physical interaction of CN β with the unfolded protein response (UPR) sensor, protein kinase RNA-like endoplasmic reticulum kinase (PERK). In accordance, induction of CN β resulted in oligomerization and activation of PERK. Strikingly, the presence of a phosphatase inhibitor did not interfere with CN β -mediated activation of PERK, suggesting a hitherto undiscovered non-enzymatic role for CN β . Importantly, the cytoprotective function of CN β was PERK-dependent both *in vitro* and *in vivo*. Loss of CN β *in vivo* resulted in a significant increase in cerebral damage, and correlated with a decrease in astrocyte size, PERK activity and glial fibrillary acidic protein (GFAP) expression. Taken together, these data reveal a critical role for the CN β -PERK axis in not only prolonging astrocyte cell survival but also in modulating astrogliosis after brain injury.

Keywords

Calcium; Stress; Ischemia; Traumatic brain injury; Endoplasmic reticulum

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Conflict of interest

The authors declare that they have no conflict of interest.

1. Introduction

After brain injury, such as cerebral ischemic stroke or traumatic brain injury (TBI), the long-term fate of cells is determined not only by the brain's initial vulnerability to the primary injury, but also by its ability to mount a competent cellular defense during the secondary injury phase. The central role of calcium and its downstream targets, such as the highly conserved Ca^{2+} /calmodulin-dependent serine/threonine protein phosphatase, calcineurin (CN) has been particularly studied in neurons. CN exists as a heterodimer consisting of a catalytic subunit (CN-A) and a regulatory subunit (CN-B) (Klee & Krinks, 1978). CN-A is encoded by three genes (PPP3CA, PPP3CB and PPP3CC genes); the products of PPP3CA and PPP3CB are CN-A isoform α and β , which share 81% sequence homology and are highly abundant in mammalian brain tissue (Kuno et al., 1992), particularly in the striatum and the CA1 region of the hippocampus (Polli et al., 1991). Here, we will refer to CN-A α /B and CN-A β /B as CN α and CN β , respectively.

Hyperactivation of CN, due to chronic and/or exacerbated cytosolic Ca^{2+} increases, has been demonstrated to mediate L-glutamate induced hippocampal neuron death by dephosphorylating the pro-apoptotic Bcl-2-associated death promoter (BAD) (Wang et al., 1999). Indeed, multiple neurodegenerative disorders have been associated with low levels of phosphorylated BAD (Reese et al., 2008; Agostinho et al., 2008). However, the role of individual calcineurin isoforms in astrocytes is unclear.

Located between blood vessels and neurons, astrocytes make crucial contributions to the normal function of the central nervous system (CNS) (Nedergaard, 1994; Trendelenburg & Dirnagl, 2005). In addition, astrocytes respond to different kinds of CNS insults, such as infections, trauma, and ischemia by a process named reactive astrogliosis, (Kurz et al., 2005). Interestingly, CN β was found up-regulated in reactive astrocytes of *Mongolian gerbils* after transient ischemia (Hashimoto et al., 1998); however, the functional impact of CN β in astrocytes has not yet been determined.

The endoplasmic reticulum (ER) is a major signal transduction organelle essential for protein folding and assembly (Ellgaard & Helenius, 2003) as well as the regulation of intracellular Ca^{2+} stores (Brostrom & Brostrom, 2003). Transient cerebral ischemia is known to impair ER function by disturbing ER Ca^{2+} homeostasis and by depriving cells of energy (Paschen, 2004). ER stress activation during acute brain damage has also been documented after TBI (Krajewska et al., 2011; Farook et al., 2013). An immediate response to ER stress is the attenuation of protein translation *via* dimerization and autophosphorylation of protein kinase RNA-like endoplasmic reticulum kinase (PERK), which subsequently phosphorylates eukaryotic initiation factor 2 alpha (eIF2 α) (Kaufman, 2004; Ma et al., 2002; Ron & Walter, 2007). The central role of ER stress and in particular, PERK, in transient cerebral ischemia is supported by an observed increase in the phosphorylation of PERK but not of other eIF2 α kinases in cortex and hippocampus (Kumar et al., 2001; Kumar et al., 2003; Owen et al., 2005). We have previously shown that the PERK arm of the unfolded protein response (UPR) is activated in astrocytes within 30 min of oxygen glucose deprivation (OGD) *in vitro* (Bollo et al., 2010). However, the precise

molecular mechanisms of PERK regulation in astrocytes after acute brain injury are unknown.

Contrary to previous studies that have linked exacerbated and prolonged cytosolic Ca^{2+} release to CN over-activation and neuronal death, we demonstrate here that $\text{CN}\beta$ promotes cell survival after acute brain injuries. Our evidence indicates that this is due to an interaction of $\text{CN}\beta$ with PERK, which is significantly increased by moderate cytosolic Ca^{2+} release in astrocytes during ER stress. Moreover, we demonstrate that the cytoprotective effect of $\text{CN}\beta$ is *in vivo* linked to PERK activity and correlates with reactive astrogliosis.

2. Results

2.1. $\text{CN}\beta$, but not $\text{CN}\alpha$, is significantly increased in astrocytes after traumatic brain injury

We first examined whether the $\text{CN}\beta$ isoform was up-regulated in astrocytes *in vivo* after a traumatic brain injury (TBI). TBI was induced using a controlled closed cortical impact (CCCI) model in the left parietal cortex (ipsilateral) without touching the right cortex (contralateral) (Fig. 1G) (Talley Watts et al., 2013). We observed low levels of $\text{CN}\beta$ expression that colocalized with glial fibrillary acidic protein (GFAP), considered to be expressed mainly by astrocytes (Fig. 1C). At 24 h post-TBI, the ipsilateral hippocampus showed a remarkable increase in $\text{CN}\beta$ expression in astrocytes, compared to the contralateral side or the sham group (Fig. 1A–C, H). In contrast, the same brain slices did not stain positive for $\text{CN}\alpha$ in astrocytes in both sides of the brain, corroborating the *in vivo* isoform cell specificity (Fig. 1D). Additionally, the expression of $\text{CN}\alpha$ did not change in response to TBI (Fig. 1D–F, I).

Perturbation of Ca^{2+} homeostasis has been implicated in the pathophysiology of cerebral ischemia and other brain injuries (Paschen, 2004). To recapitulate the *in vivo* situation, we used the ER Ca^{2+} -ATPase inhibitor, thapsigargin (Tg), to reduce ER Ca^{2+} stores and induce ER stress (Bollo et al., 2010; Thastrup et al., 1990). C8D1A cells (type I astrocyte cell line) were treated with 1 μM Tg for 1 h. Immunoblots showed that $\text{CN}\beta$ levels significantly increased by 2-fold as compared to dimethylsulfoxide (DMSO, Con) treatment (Supplementary Fig. 1A, B). However, the levels of the α isoform of CN remained unchanged (Supplementary Fig. 1A, B). We also determined CN expression after OGD. Again, $\text{CN}\beta$ levels were significantly increased in mouse primary astrocytes after 30 min (Supplementary Fig. 1C, D), suggesting a role for $\text{CN}\beta$ under conditions of astrocyte acute stress.

2.2. $\text{CN}\beta$ null mice exhibit higher lesion volume and increased cytotoxic edema after TBI

To further determine the potential role of $\text{CN}\beta$ in acute brain injury, we compared the outcome of CCCI induced TBI on $\text{CN}\beta$ wildtype ($\beta^{+/+}$) and knockout ($\beta^{-/-}$) mice. Lack of $\text{CN}\beta$ protein in null mice was confirmed by immunoblotting (Klee et al., 1998). $\text{CN}\alpha$ expression was clearly detected in mice of both genotypes (Fig. 2B) (Wu et al., 2004). At 24 h post-TBI, Nissl staining showed significantly increased lesion volume in $\beta^{-/-}$ mice by over 2-fold compared with lesion volume in $\beta^{+/+}$ mice (Fig. 2A, C). $\beta^{-/-}$ mice also demonstrated a remarkable increase in the soma size of neurons in both the ipsilateral cortex and

hippocampal CA1 region as compared to $\beta^{+/+}$ mice (Fig. 2D–G). Together, these data suggest that loss of CN β significantly worsens the outcome of acute brain injury.

2.3. CN β physically interacts with PERK and promotes PERK oligomerization and phosphorylation in stressed astrocytes

We show here that conditions that upregulated CN β in C8D1A astrocytes (Supplementary Fig. 1) also increased the levels of phosphorylated PERK (P-PERK) and eIF2 α (P-eIF2 α) (Fig. 3A, B) (Mounir et al., 2011). Protein lysates from C8D1A cells exposed to 1 μ M Tg for 1 h and probed for PERK and eIF2 α on immunoblots were found to have significantly increased expression of the phosphorylated forms of PERK and eIF2 α (Fig. 3 A , B). Immunostaining also demonstrated increased P-PERK in astrocytes exposed to Tg (Supplementary Fig. 2A – C). Consistently, OGD also increased P-PERK expression in primary mouse astrocytes (Supplementary Fig. 2D–F). Taken together, our results indicate that induction of CN β is correlated with phosphorylation of PERK and eIF2 α in astrocytes in conditions that mimic ischemic stress *in vivo*.

The association of the α isoform of CN (CN α) and PERK under acute ER stress has been studied in *Xenopus* oocytes, but CN β was not detected in these cells (Bollo et al., 2010). Both our *in vitro* and *in vivo* data suggest that CN β is the major isoform of CN in stressed astrocytes. Given that P-PERK was enhanced in astrocytes during ER stress, we hypothesized that CN β directly interacted with PERK to modulate cell stress that occurs after brain injury (Nakka et al., 2014). To test this hypothesis, we examined whether endogenous CN β interacted with endogenous PERK in stressed primary astrocytes by co-immunoprecipitation. As shown in Fig. 3 (C, D), increased P-PERK was bound to CN β on treatment of primary astrocytes with Tg. Next, we performed pull-down assays using purified recombinant GST-cytosolic PERK (GST-cPERK) and either His-CN α or His-CN β (Fig. 3E, F). We note that all of the pull-down assays were performed using the same stringency conditions (high ionic strength and in the presence of detergent). These experiments revealed that the association between CN β and GST-cPERK was significantly weaker than the CN α /PERK interaction. Finally, we performed an *in vitro* kinase assay to determine the functional consequences of these interactions. To do this, the same recombinant proteins were incubated with [γ ³²P] ATP (Fig. 3G, H). We found that the β isoform was able to promote PERK autophosphorylation at a level significantly higher than the α isoform. These data demonstrate that the efficacy for promoting PERK phosphorylation did not necessarily depend on the strength of interaction.

We also investigated the potential effect of the CN regulatory subunit (CN-B) on PERK interaction and auto-phosphorylation (Supplementary Fig. 3). However, in order to detect a trend in specific interaction with GST-cPERK compared with GST only (negative control; Supplementary Fig. 3A, B), we required a 1000-fold higher concentration of CN-B compared to the association with the heterodimer (subunits A and B, Fig. 3E, F). Interestingly, CN-B was unable to promote GST-cPERK auto-phosphorylation even when added at a concentration higher than the heterodimer (Supplementary Fig. 3C, D).

Overall, these data demonstrate that direct interaction of CN β /PERK promotes PERK phosphorylation in astrocytes during acute ER stress. It has been proposed that formation of

PERK oligomers is a step that precedes trans-autophosphorylation (Polley et al., 2013; Bertolotti et al., 2000) Therefore, we tested if CN β promotes PERK cluster formation or alternatively if CN β interacts with PERK after PERK has oligomerized. We incubated GST-cPERK with increased concentrations of CN β and a crosslinker, disuccinimidyl suberate (DSS). The complex was run on a gel under reducing conditions and probed with an anti-PERK antibody (Fig. 3I). In solution, GST-cPERK existed not only as monomers but also as stable dimers and oligomers. This was likely due to dimerization of the GST portion of GST-cPERK as reported by several groups (Parker et al., 1990; Ji et al., 1992; Welihinda & Kaufman, 1996) and also due to the presence of a dimerization subdomain located in the cytosolic domain of PERK (Yamani et al., 2014). We observed that increasing concentrations of CN β resulted in more dimers and oligomers of GST-cPERK. The same crosslinking assay was performed on purified CN β and maltose binding protein (MBP)-cPERK, in which MBP is fused with His-cPERK to increase protein solubility. Similar to GST, MBP portions dimerized by themselves as reported previously (Richarme, 1982). As with GST-cPERK, we also observed increased dimers and oligomers of MBP-cPERK with increasing concentration of CN β (Supplementary Fig. 3E). We note that the difference in the molecular weight of the PERK monomers is due to the fusion tags to the proteins (~25 KDa and 42 KDa for GST and MBP, respectively). In another set of experiments, we cross-linked GST-cPERK with 1.2 μ M of CN β . Immunodetection using anti-CN β and anti-PERK antibodies confirmed increased level of high molecular weight CN concurrently with the oligomerization of GST-cPERK and that the presence of CN induced PERK oligomerization (Fig. 3J). We note that the phosphorylated form of PERK is expected to run as a heterogeneous population (smear), because of the many phosphorylation sites in the PERK protein (Volmer et al., 2013). Therefore, CN β participates in PERK cluster formation and we conclude that CN β acts as a cytosolic ligand to promote PERK oligomerization, favoring PERK trans-autophosphorylation and its kinase activity.

(D) Quantification of P-PERK/T-PERK densitometric ratio in (C) ($n = 3$, mean \pm SEM, $*p < 0.05$ by unpaired two-tailed Student's *t*-test). (E) GST *pull-down* assay with either 8 nM of CN α or CN β . The proteins were incubated with glutathione sepharose 4B for 1 h, resolved on a 12% SDS-polyacrylamide gel and probed with an anti-calceineurin PAN-A antibody. CN *pull-down* levels are shown for GST alone and GST-cPERK. (F) Densitometric histogram of (E) ($n = 3$, mean \pm SEM, $**p < 0.01$ by unpaired two-tailed Student's *t*-test). (G) GST-cPERK was added to all reaction mixtures along with [γ ³²P] ATP in the absence or the presence of either of 0.043 μ M of CN α or CN β . Reaction mixtures were run on SDS-PAGE and visualized by autoradiography. (H) Quantification of cPERK auto-phosphorylation density ($n = 5$, mean \pm SEM, $*p < 0.05$ by one-way ANOVA). (I) Recombinant His-CN β and GST-cPERK were incubated at the concentrations indicated, in the presence of 0.3 mM of DSS cross-linker for 30 min at room temperature. The ensuing protein complexes were run on SDS-PAGE and detected by immunoblotting using an anti-PERK antibody. (J) Recombinant His-CN β (1.2 μ M) and GST-cPERK (0.01 μ M) were incubated in the presence of 0.3 mM of DSS for 30 min at room temperature. The protein complexes were run on SDS-PAGE and detected by immunoblotting using antibodies against CN β and PERK.

2.4. Chemical or light induced-dimerization induces immediate CN β /PERK interaction in live cells, resulting in PERK phosphorylation

To determine if the observed CN β and PERK interaction *in vitro* also occurred in live cells, we took advantage of the rapamycin-driven heterodimerization technique (Komatsu et al., 2010) to confirm the effect of acute CN-PERK interaction in live cells (Komatsu et al., 2010). This module was based on the rapamycin-induced FK506 binding protein (FKBP)/FKBP-rapamycin binding domain (FRB) interaction. For these experiments, the effector (FKBP-YFP-CN β) and anchor (FRB-CFP-Cb5) units were overexpressed in human astrocytes. The anchor unit has the trans-membrane domain of cytochrome b5, an ER membrane protein, tail-anchored to the cytoplasmic face of the ER (Supplementary Fig. 4A) (Honsho et al., 1998); thus, we were able to observe the ER structure with CFP and cytosolic distribution of YFP (Fig. 4E, H). After addition of 100 nM rapamycin, FKBP-YFP-CN β was recruited to the ER, where FRB-CFP-Cb5 was located (Fig. 4C). Immunostaining showed that cells with CN β translocation exhibited a significant increase in P-PERK fluorescence intensity (Fig. 4D, K). However, in DMSO (vehicle)-treated cells, neither translocation of CN β nor changes in P-PERK was observed (Fig. 4A, B, K). Together, these data reveal that recruitment of CN β to the ER membrane in live cells facilitates CN β -PERK interaction and leads to PERK autophosphorylation.

Previous studies have shown that intracellular Ca²⁺ levels can affect PERK and CN dynamics (Bollo et al., 2010; Wang et al., 2013). Therefore, we tested the effect of intracellular Ca²⁺ levels on CN-mediated PERK activation. We added a very low concentration of Tg to induce Ca²⁺ release (Supplementary Fig. 4B) (Kovacs et al., 2005). DMSO with Tg caused neither the translocation of CN β in YFP nor significant changes in P-PERK intensity (Fig. 4F, G, K), suggesting that at low concentration of Tg, the moderate cytosolic Ca²⁺ increase is not sufficient to have effect on PERK phosphorylation by itself. However, cells with both rapamycin and Tg treatment showed co-localization of YFP-CN β with the CFP-ER anchor. These cells also displayed increased P-PERK compared with non-transfected neighbor cells (Fig. 4I–K). P-PERK intensity was increased by 30% in cells that received both rapamycin and Tg, compared with the rapamycin-only treatment group (Fig. 4K). These results are consistent with previous data from our group (Bollo et al., 2010; Paredes et al., 2013) and indicate that Ca²⁺ release from ER enhances PERK activation.

2.5. CN β induces PERK phosphorylation independent of its phosphatase activity

To determine if the enzymatic activity of CN β was required or altered in mediating its effects on PERK phosphorylation, we constructed a pUltra-lentiviral vector to overexpress CN β in cultured astrocytes. Immunoblots showed a 2-fold increase in the levels of CN β in lenti-GFP (CN β) transduced astrocytes and correspondingly, increased P-PERK levels compared with lenti-GFP (control) transduced cells (Supplementary Fig. 5). Similar to the results from western blots, an immunocytochemistry assay showed that CN β levels were increased in cultured astrocytes infected with lentivirus lenti-GFP (CN β) compared to those infected with lenti-GFP (Fig. 5A, B, G). The levels of P-PERK were increased by over 5-fold in the astrocytes that overexpressed CN β (Fig. 5C, D, H). Next, we exposed these astrocytes to quercetin (QC), a novel non-competitive CN inhibitor with strong phosphatase inhibitory activity (Wang et al., 2010; Lei et al., 2011). Interestingly, the presence of the CN

inhibitor did not interfere with PERK phosphorylation in cells overexpressing CN β ($n = 3$, $***p < 0.001$) (Fig. 5E, F, H). These data strongly suggest that the effect of CN β on PERK phosphorylation is independent of its phosphatase activity.

2.6. PERK is required for CN β -mediated protection of astrocytes

We also investigated the physiological consequences of increased expression of CN β in ER-stressed astrocytes from both PERK null mice (*Perk*^{-/-}) and wild type siblings (*Perk*^{+/+}). CN overexpression by lentiviral infection did not cause significant changes in cell viability of *Perk*^{+/+} and *Perk*^{-/-} astrocytes at rest (Fig. 6A, C, F, H; Supplementary Fig. 6A – C). Treatment of pUltra lentivirus transduced cells with *tert*-butyl hydroperoxide (tBuOOH) (He et al., 2008) for 2 h or OGD for 1 h showed that overexpression of CN β increased the percentage of live cells from 42% to 51% (Fig. 6A, B, E) and from 50% to 69% (Fig. 6F, G, J), respectively. However, overexpression of CN β in *Perk*^{-/-} astrocytes had no significant effect on cell survival (Fig. 6C–E, H–J). These findings strongly indicate that lack of PERK abolishes the protective effect of CN β on stressed astrocytes.

In a similar experiment, we overexpressed CN β in astrocytes using transient transfection and exposed these cells to tBuOOH for 2 h. We observed 50% cell death in untransfected cells (Con) whereas overexpression of CN β reduced cell death by 25% ($n = 4$, $**p < 0.01$) (Supplementary Fig. 6D, E). More interestingly, there was no significant difference in viability of *Perk*-deficient cells with overexpression of CN β as compared to control cells.

2.7. PERK is required for CN β -mediated protection from brain injury in vivo

To determine the involvement of PERK in CN β -mediated protection from acute brain injury, we measured the ischemic outcome of mice with pharmacologically inhibited PERK activity. GSK2656157 is a potent and selective inhibitor of PERK kinase (Axten et al., 2012). We injected GSK2656157 intraperitoneally into wildtype (β ^{+/+}) mice. Immunoblots of brain tissue showed that P-PERK and P-eIF2 α were significantly inhibited at 12 h after administration of PERK inhibitor (Fig. 7A, B). At this time point, we induced a photothrombotic stroke in mice using Rose-Bengal (RB) dye (Fig. 7C). Fluorescence gradually disappeared in the target vessel, suggesting that a stable clot was formed (Fig. 7C). After 24 h, brain slices were stained with the vital dye, 2,3,5-triphenyl-tetrazolium chloride (TTC) (Fig. 7D). The absence of staining indicated necrotic tissue and this was used to define the area of brain infarction. The average infarct volume in β ^{+/+} mice was 3.61 ± 0.88 mm³. This volume was significantly increased by two-fold when PERK activity was inhibited (Fig. 7E).

To investigate the role of PERK-CN β interaction after brain ischemia, we first measured cerebral damage in β ^{-/-} mice after acute stroke. This average infarct volume in β ^{-/-} mice was 10.54 ± 2.11 ($n = 5$) mm³, which is significantly larger than that in β ^{+/+} mice (Fig. 7D, E). Importantly, administration of PI did not change the infarct size in mice deficient in β ^{-/-} mice (Fig. 7D, E).

In parallel, we directly injected the PI into the lateral ventricle, as illustrated by Evans blue staining (Supplementary Fig. 7A). Significant decreases in P-PERK and P-eIF2 α levels were observed at 12 h after micro-injection of PI (Supplementary Fig. 7 B, C). RB-induced

photothrombosis was performed on the contralateral side of micro-injection (Supplementary Fig. 7D), resulting in a doubled stroke volume in PI-treated group compared to Con group (Supplementary Fig. 7E, F). Taken together, these data strongly suggest that PERK activity is important for protection following acute cerebral ischemia and that CN β and PERK might function through the same cytoprotective pathway after ischemia.

Related to these data we also discovered that in $\beta^{+/+}$ mice, GFAP expression and the size of astrocytes near the infarct were significantly increased in the ipsilateral side of stroke as compared to the contralateral side, indicating increased proliferation of reactive astrocytes (Stichel & Muller, 1998) (Fig. 8A, B, G). We also noticed a significant increase in phosphorylation of eIF2 α after stroke in the ipsilateral sides in $\beta^{+/+}$ mice as compared to contralateral sides, strongly suggesting activation of the CN β – PERK pathway (Fig. 8C, H). However, compared to $\beta^{+/+}$ mice, $\beta^{-/-}$ mice exhibited much lower levels of GFAP and P-eIF2 α in reactive astrocytes as well as considerably smaller astrocytes after stroke (Fig. 8G – J). We note that GFAP expression was still significantly increased after stroke in the ipsilateral sides of $\beta^{-/-}$ mice, while P-eIF2 α levels and astrocytes size were not increased when compared to the contralateral sides (Fig. 8E, F, G, H). These data indicate that up-regulation of CN β protected against ischemic insult by enhancing PERK signaling in astrocytes, which is associated with increased overexpression of GFAP and the astrocytes size, both characteristic of reactive gliosis.

3. Discussion

In this study, we identify an unexpected protective role for CN β in astrocytes *in vivo* using two acute brain injuries models: TBI and stroke. We show that CN β directly interacts with PERK, promoting its dimerization/oligomerization and auto-phosphorylation, and that Ca²⁺ appears to function as a co-factor. Importantly, we demonstrate for the first time, a critical role for the β isoform of CN in cell survival after brain injury, an observation that contrasts with the established role of CN in neuronal cell death (Shioda & Fukunaga, 2011). Moreover, we show that the protective effect of CN β appears independent from its known phosphatase activity. Finally, our data show that activation of the CN-PERK-eIF2 α pathway correlates with GFAP expression, suggesting this pathway may also directly regulate reactive gliosis signaling.

Our finding that CN β levels are increased in reactive astrocytes in the TBI mouse model is consistent with a previous report showing that CN β was up-regulated in reactive astrocytes in ischemic hippocampal regions of Mongolian gerbils (Hashimoto et al., 1998). In contrast, it has also been reported that CN levels do not change after trauma (Kurz et al., 2005). These differences may be due to the fact that these authors used only western blots to measure CN levels and that the antibody used did not distinguish between CN isoforms. Given that CN α is more abundant than CN β in mammalian brain (Kuno et al., 1992), we speculate that the whole brain homogenate analysis may have obscured smaller, astrocyte-specific changes in CN β .

More importantly, our results also revealed that acute brain injury caused more damage in mice lacking CN β . This is in agreement with our *in vitro* data showing a protective benefit

of overexpressing CN β in astrocytes. The cell survival role of CN β in astrocytes is in contrast to the apoptotic role of CN when over-activated by chronic and exacerbated cytosolic Ca²⁺ increase in hippocampal neurons (Wang et al., 1999). This cytoprotective function also appears to be distinct from another reported role of CN in reactive astrocytes, where CN significantly diminishes inflammatory injury by its phosphatase activity and subsequent signaling of NFAT (nuclear factor of activated T) cells (Fernandez et al., 2007; Pyszynska et al., 2001). This conclusion is based on our observation that CN β promoted PERK phosphorylation in the presence of a phosphatase inhibitor.

In vitro experiments using recombinant proteins revealed that CN β binds to the cytosolic domain of PERK with lower affinity than CN α , however, CN β results in markedly higher PERK auto-phosphorylation. The regulatory subunit B of CN is unable to promote PERK auto-phosphorylation. This observation, as well as the difference in PERK binding affinity of the CN isoforms, argue against non-specific effects. Moreover, direct binding of CN β to the higher oligomers of GST-cPERK suggests that CN β drives PERK clustering, phosphorylation, and activation of its kinase activity. Signaling outputs from another ER stress sensor, IRE1 α , have been reported to vary with its oligomerization state (Han et al., 2009). In a similar fashion, CN β may act as a cytosolic regulator of PERK activity that helps tone the intensity of stress stimuli by modulating PERK oligomerization.

We found that CN β -PERK interaction occurs within 30 min of ER stress induced by Tg or OGD treatments. We also note that CN β levels significantly increased in cultured astrocytes under the same conditions. The speed and selectivity of this response, confirmed using chemical-activated dimerization techniques, appears to mimic an acute stress response. These data suggest that CN β and PERK are only required to be in close proximity to promote PERK autophosphorylation. Since low doses of Tg further increase the efficacy of chemical dimerization, we suggest that Ca²⁺ acts as a PERK cofactor on the cytosolic side of the ER, in agreement with previous findings of our group (Bollo et al., 2010; Paredes et al., 2013).

Moreover, we found that acute brain injury causes more damage in mice when PERK activity is pharmacologically inhibited, which is consistent with published reports showing loss of PERK-enabled neurons more sensitive to stress and decreases viability, probably because the cells are unable to mount an appropriate protective response (Ryu et al., 2002). However, it has also been reported that synaptic failure and neuronal death are associated with persistent global protein synthesis inhibition that is mediated by over-activation of PERK in a prion-diseased mouse model (Moreno et al., 2012; Moreno et al., 2013). It is widely assumed that the duration and/or strength of PERK signaling helps determine cell fate after stress, either leading to cell survival or death (Lin et al., 2009). In response to stress, transient PERK-eIF2 α signaling acutely attenuates overall protein synthesis to provide time to resolve and alleviate stress. If unsuccessful, the cell progresses to cell death by inducing the transcription of CHOP that is downstream of PERK-eIF2 α pathway (Zinszner et al., 1998; Oyadomari et al., 2002; Pennuto et al., 2008). PERK is well-known to be acutely phosphorylated in neurons within 20 min of ischemia (Kumar et al., 2001). However, our *in vivo* data were obtained 24 h post-stroke and showed that phosphorylation of eIF2 α was still significantly increased in reactive astrocytes (Fig. 8). Our data show that

eIF2 α can remain phosphorylated in astrocytes for prolonged periods without inducing cell death. This holding pattern may represent a different phase or steady-state level of stress mediated by CN β . It is possible that this long-term, pro-survival stress is specific to CN β in astrocytes and the isoform-specific interaction of CN/PERK is critical in the outcome of acute brain injury.

As expected, GFAP expression was also significantly increased in WT mice after stroke, but to a much lower extent in $\beta^{-/-}$ mice. GFAP is considered to be a signaling platform that coordinates appropriate responses in situations related to cellular stress (Kurz et al., 2005). We note that acute brain injury resulted in less reactive gliosis (Tajiri et al., 2004) but larger infarct volumes (Gotoh et al., 2002) in GFAP null mice compared to controls. On the other hand, several reports have also found a link between CN expression levels and reactive gliosis (Norris et al., 2005; Abdul et al., 2009; Lim et al., 2013). Our data further indicate that mice deficient in CN β exhibit disrupted P-eIF2 α , which constitutes the first molecular evidence that the CN β – PERK pathway could represent an active link between the UPR and reactive gliosis activation. Signaling induces global translation inhibition, but also stimulates the expression of a group of genes *via* the transcription factor, ATF4, when eIF2 α is phosphorylated (Lu et al., 2004). Therefore, we speculate that CN β – PERK – eIF2 α pathway positively modulates GFAP expression during astrogliosis induced by acute brain injury.

Overall, our data identify a novel cytoprotective role for the ubiquitous protein, CN β , which surprisingly appears independent of its phosphatase activity. Our findings also suggest that this signaling pathway may modulate astrogliosis *via* the PERK – eIF2 α arm of the integrated stress response/UPR pathway. We anticipate that molecular interventions could be developed that either promote or mimic CN β binding to PERK, which, in turn, could be used as a potent therapeutic strategy in patients suffering from either trauma or ischemic insults.

4. Material and methods

4.1. Animals, human tissue, and cell cultures

CN β heterozygotes on C57BL/6 background were gifts from Dr. Hanna Abboud (University of Texas Health Science Center at San Antonio). Male CN β knockout mice and wild-type littermates were investigated at 8–10 weeks of age. *Perk* heterozygotes on C57BL/6 background were purchased from the Jackson Laboratory (STOCK *Eif2ak3^{tm1Dron}/HotaJ*) and originally generated by David Ron's laboratory (University of Cambridge) (Harding et al., 2001). Neonates of *Perk* knockout and wild-type littermates as well as adult male C57BL/6 (4– 6 month mice) were used for primary astrocytes culture. All procedures that involved animal subjects were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio.

Human astrocytes were cultured from human brain tissue of a 40 year-old male patient and maintained using the same protocols as mouse astrocyte cultures. The patient who gave his informed consent to the Department of Neurosurgery at the University of Texas Health Science Center San Antonio. The protocols were approved by the local Ethics committee.

Primary astrocyte cultures were prepared as previously described (Lin et al., 2007). The C8D1A astrocyte cell line was purchased (#CRL-2541; ATCC) and maintained in DMEM (#30-2002; ATCC) containing 10% FBS. All cell cultures were incubated at 37 °C in a humid atmosphere containing 5% carbon dioxide (CO₂).

4.2. Immunostaining

We performed immunostaining on cells grown on glass coverslips and fixed with 4% paraformaldehyde or on brain sections (30 μM) as previously described (Holguin et al., 2004). For quercetin (QC, #Q4951; Sigma) treatment, cells were exposed to 40 μM QC or DMSO for 20 min before fixation. Primary antibodies used were rabbit anti-phosphorylated PERK (conjugated to Alexa 647, #bs-3330R-A647; Bioss), rabbit anti-CNα (#13422-1-AP; Proteintech), rabbit anti-CNβ (#07-1439; Millipore), rabbit anti-phosphorylated eIF2α (P-eIF2α, #μA5 – 15133; Thermo scientific) and mouse anti-GFAP (#MAB 360; Millipore). Secondary antibodies (Life Technologies) were added as appropriate. Samples were imaged on an inverted microscope (Nikon TE300, Nikon Instruments Inc.) or a confocal microscope (Olympus FV1000, Olympus; Zeiss LMS 710, Zeiss). The immunofluorescence intensity for each cell was analyzed by NIH Image J software. >10 cells were analyzed in every independent experiment, which was replicated 3 times. For sections, corrected total cell fluorescence intensity was calculated by measuring integrated density minus background reading.

4.3. Immunoprecipitation and co-immunoprecipitation

Cell lysates were incubated with 0.3 mM DSS for 30 min, and microsomal fractions were obtained as described previously (Bollo et al., 2010). Anti-CNβ (5 μg) (#07-068; Millipore, (25) was added. After incubation overnight, the immune complex was washed and the eluted proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and detected by immunoblotting with anti-PERK (PERKUT).

4.4. In vitro kinase activity assay and pull-down assay

In vitro kinase activity assay and pull-down assay were performed as described previously (Bollo et al., 2010).

4.5. Western blot

Western blot analysis was performed using 30 μg of total protein in cell lysates. The following antibodies were used in this study: anti-CNα (#07-067; Millipore), anti-CNβ (#07-068; Millipore), anti-actin (#MAB1501; Millipore), anti-CN-B (#07-069; Millipore), and anti-GFP (#111258; Abcam), anti-P-PERK (#3179; Cell signaling Technology), anti-T-PERK (#3192; Cell signaling Technology), anti-P-eIF2α (#3597; Cell signaling Technology), and anti-T-eIF2α (#9722; Cell signaling Technology).

4.6. ER tracker imaging

Cultured human astrocytes expressing CFP-FRB-cb5 were washed with HBSS and then loaded with 1μM of ER-tracker™ blue-white DPX (#E-12353; Life technologies) for 30 min

at 37 °C. After incubation, cells were washed with HBSS and then observed using a Nikon 60 × oil immersion objective (NA 1.2) on Olympus FV-1000 confocal microscope.

4.7. Measurement of Ca²⁺ signal

Human astrocytes were incubated with the mixture of Fluo-4 AM (#F14217; Life technologies) and pluronic acid F-127 (#P3000MP; Life technologies) for 30 min at the room temperature. Cells were washed out with PBS and then incubated for additional 30 min to allow complete de-esterification of intracellular AM esters. After incubation, cells were treated with 25 nM of Tg. Confocal microscopy images were acquired every 5 min for 30 min using Nikon 60× oil immersion objective (NA 1.24) under the Nikon swept field confocal microscope (Nikon instruments Inc., Melville). F is the mean fluorescence intensity of Fluo-4 measured throughout the regions of interest (ROIs). F_0 represents the intensity at the time 0 and ΔF represents a change in intensity at different time points compared to the time 0. At least 3 cells were analyzed in each independent experiment, which was replicated for three times.

4.8. Stereotaxic injection into the mouse lateral ventricle

Male B6 Albino mice (8 weeks, Charles River laboratories) were anesthetized with isoflurane (3% induction and 1% maintenance; #NDC 13985-030-60, Vetone,) in 100% oxygen and placed into a stereotaxic frame. The skull was exposed, a hole was drilled, and 1 μ l of Evans (#E2129; Sigma) was injected into the left lateral ventricle, using a 10 μ l Hamilton syringe to establish our initial coordinates. The following stereotaxic coordinates were used: -1.0 mm anterior-posterior (AP), -1.0 mm medial-lateral (ML) and -2.0 mm dorsolventral (DV) to the bregma (Stanley et al., 2004). Using the same coordinates, a total volume of 1 μ l of GSK2656157 (2 mM in DMSO, #406230; MedKoo Bio-science) or DMSO (Con) was injected into the lateral ventricle.

4.9. Traumatic brain injury (TBI) and Nissl staining

A controlled closed cortical impact (CCCI) model was used to generate a moderate TBI as previously described (Talley Watts et al., 2013). In brief, mice were anesthetized, the skull exposed and a pneumatic impact device was used to produce a moderate TBI over the somatosensory cortex (Talley Watts et al., 2013). The calibrated impact was 4.5 m/s and a depth of 2 mm. Scalp incisions were closed and mice were monitored until fully awake. Twenty-four hours after the initial trauma, mice were perfused with 4% paraformaldehyde (#RT157-4; Electron Microscopy Sciences) before brains were harvested and sectioned. Nissl staining was used to reveal histology of the cortical lesion area as described previously (Talley Watts et al., 2013). Lesion volume was calculated as the sum of lesion areas in consecutive sections over the rostro-caudal extension of the brain. Image J was used to determine the area of each soma.

4.10. Cortical photothrombosis and Triphenyl-tetrazolium chloride (TTC) staining

Photothrombotic stroke was induced by focusing light on the cortex of Rose Bengal (RB; #330000; Sigma) tail-vein injected mice (Zheng et al., 2010). TTC (#17779 FLUKA; Sigma) staining was used to evaluate the infarct size at 24 hour post-RB-induced-

photothrombosis (Zheng et al., 2010). Infarct volume was calculated by multiplying the sum of the infarcted areas (mm^2) of various sequential coronal sections by slice thickness (1 mm).

4.11. ER stress induction

Thapsigargin (1 μM) (Tg, #T9033; Sigma), *tert*-butyl hydrogen peroxide (100 μM) (tBuOOH; #B-2633; Sigma), and oxygen glucose deprivation (OGD) were used as stressors in this study. OGD was performed as previously described (Badiola et al., 2011). Briefly, cultured primary astrocytes were washed twice, incubated in glucose-free DMEM, (#11966025; Life Technologies) and transferred to an anaerobic chamber filled with a gas mixture of 95% nitrogen and 5% CO_2 at 37 °C.

4.12. Recombinant protein cross-linking assay

GST-cPERK, MBP-cPERK, 6His-CN β , 6His-CN α and 6His-CN-B protein purifications were performed as previously described (Bollo et al., 2010; Li & Sousa, 2012). All recombinant proteins, even at concentrations of 1.0 to 1.2 mg/ml, showed no absorbance from 310 to 340 nm in absorption spectra (220 to 350 nm), supporting the absence of aggregates in the solution. Recombinant proteins His-CN β and GST-cPERK (or MBP-cPERK) were incubated in the presence of 0.3 mM DSS cross-linker (#21658; Thermo Scientific,) for 30 min at room temperature. Reactions were quenched in 1 M tris (hydroxymethyl) aminomethane hydrochloride (Tris HCl; pH, 7.5) for 15 min at room temperature. The protein complexes were analyzed by SDS-PAGE (4% to 12%) and detected by immunoblotting either with anti-PERK antibody (PERKUT) or with anti-CN β (#07-068; Millipore).

4.13. Rapamycin-inducible dimerization assay

This assay was performed as previously described (Komatsu et al., 2010). Briefly, FKBP was fused with YFP-CN β (effector unit) and FRB was fused with CFP-cytochrome *b5* (Cb5) (anchor unit). Transfected human astrocytes were treated with 100 nM rapamycin (#9904; Cell Signaling) to induce dimerization events for 30 min. A second treatment paradigm was rapamycin (10 min) followed by Tg (25 nM; 20 min). DMSO with or without Tg was used as a control reagent. Cells were immediately fixed after 30 min of treatment and immunostained with anti-P-PERK antibody conjugated to Alexa 647 (#bs-3330R-A647; Bioss). >20 transfected cells were analyzed in each group.

4.14. Lentiviral vector production and transduction

A third generation, multicistronic lentiviral vector was purchased (pUltra plasmid 24129; Addgene). The cDNA encoding CN β was subcloned into the *XbaI/BamHI* site and the cDNA encoding CN-B was subcloned into the *NheI* site in the vector (pUltra). The constructed GFP-P2A-CN β -T2 A-CN-B can be used to express GFP, CN β , and CN-B proteins from a single open reading frame by efficient cleavage within the 2A peptide sequence.

Lentiviral vectors were produced as described previously (Zufferey et al., 1997). Briefly, pUltra-CN β -CN-B (lenti-GFP (CN β)) or pUltra control (lenti-GFP) were cotransfected with

the second generation packaging plasmid, psPAX2, and the envelope plasmid, pMD2.G using Lipofectamine LTX into HEK293FT cells. The supernatant medium was collected 4 days after transfection and concentrated (Lenti-X concentrator, #631231; Clontech) using the manufacturer's protocol. The viral particle titer was determined by transduction of HEK293 cells followed by fluorescence activated cell sorting (FACS) analysis.

Primary isolated astrocytes from *Perk*^{-/-} and *Perk*^{+/+} siblings were plated at 5000 cells per well in a 24-well plate and infected with the viral particles (multiplicity of infection, 200) in the presence of a transduction reagent (Transdux, #LV850A-1; System Biosciences) by centrifugation at 800 ×g for 1.5 h. Cells were placed in an incubator (5% carbon dioxide; 37 °C) overnight and observed every 24 h. The highest expression level of GFP was usually visualized 5 to 7 days after infection.

4.15. Cell viability assay

Sensitivity of astrocytes to oxidative stress was assessed by exposing cultures to tBuOOH for 2 h or OGD for 1 h. Cells were stained with Hoechst 33342 (10 µg/ml, #H-3570; Life Technologies) to label all cell nuclei and/or calcein red-orange (CellTrace AM, 2 µM, #C34851; Life Technologies) to identify live cells that maintained their plasma membrane integrity. Cells with good morphology were counted as live and cells with beading were counted as dead cells. For pUltra virus-infected cells, cells with GFP expression represented viable cells. Images were acquired on an inverted microscope (TE300; Nikon). We calculated percent live cells as the number of viable cells divided by the total number of cells labeled with Hoechst 33342 (#H3570; Life technologies). We counted 5 random fields in every independent experiment, which was replicated a minimum of three times.

4.16. Statistical analysis

Results are presented as means of independent replicates of experiments ± SEM. Comparisons were made with two-tailed Student's *t*-test, one-way analysis of variance (ANOVA), and Newman-Keuls test. Differences were considered as significant at $p < 0.05$, moderately significant at $p < 0.01$, or highly significant at $p < 0.001$.

The following are the supplementary data related to this article.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

UPR	unfolded protein responses
CN	calcineurin
eIF2α	eukaryotic initiation factor 2 alpha
PERK	protein kinase RNA-like endoplasmic reticulum kinase
TBI	traumatic brain injury
GFAP	glial fibrillary acidic protein
Tg	thapsigargin
OGD	oxygen glucose deprivation

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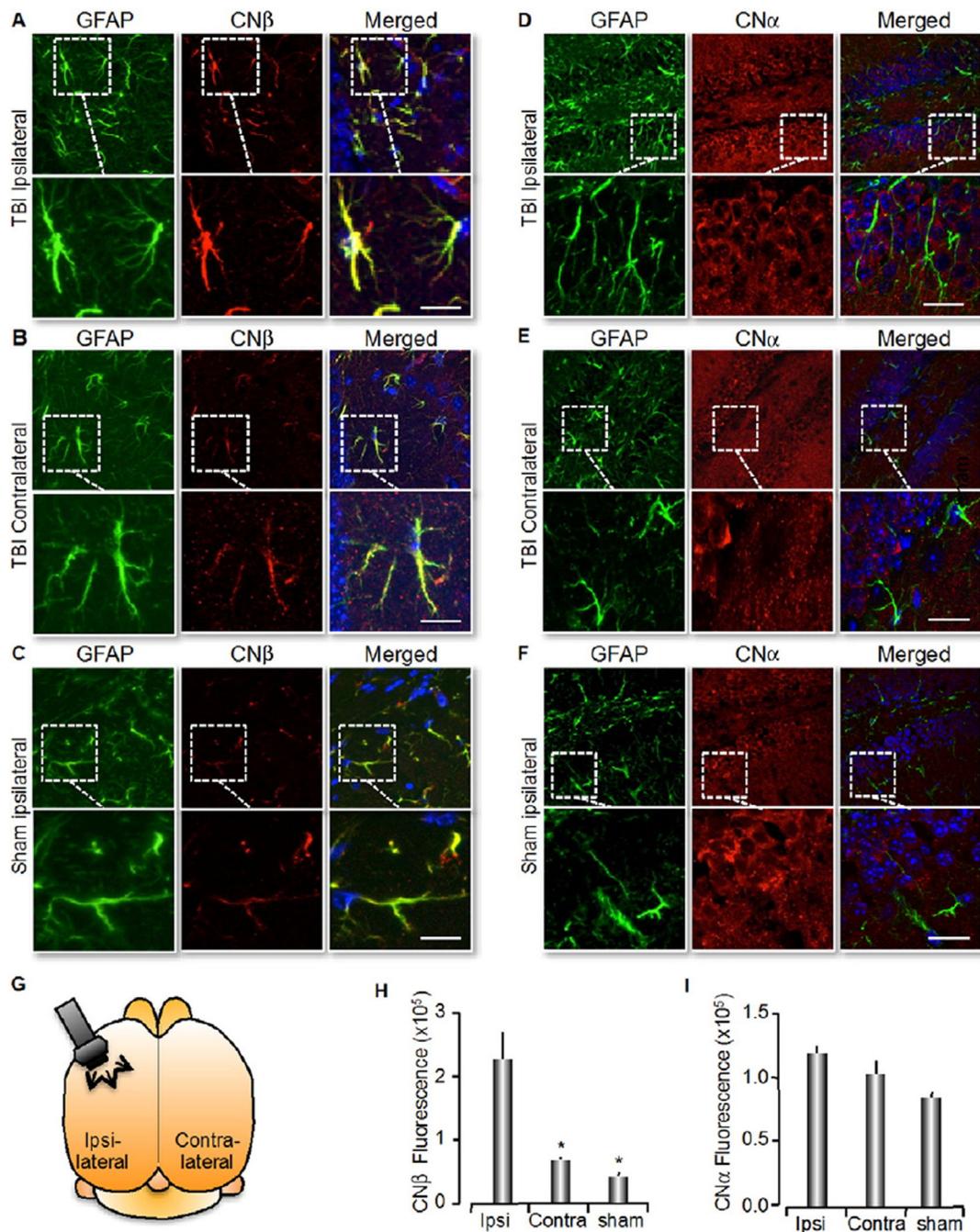


Fig. 1. CN β , but not CN α , expression increases in astrocytes *in vivo* after TBI. Representative Z-projection merged images of mice brains harvested 24 h post-TBI and stained with antibodies against CN β or CN α (red), GFAP (green) and DAPI (nuclei, blue). Images were acquired on an Olympus FV1000 confocal microscope. (A, D) Ipsilateral side of TBI brain sections; (B, E) Contralateral side of TBI brain sections; (C, F) Brain sections from sham mice. (G) Schematic diagram of brain undergoing TBI. Ipsilateral and contralateral sides of trauma are labeled. (H, I) Quantification of fluorescence intensity in panels above from 3

mice ($n = 3$, mean \pm SEM, $*p < 0.05$ by one-way ANOVA). Scale bar: 10 μm (A–C); 20 μm (D–F).

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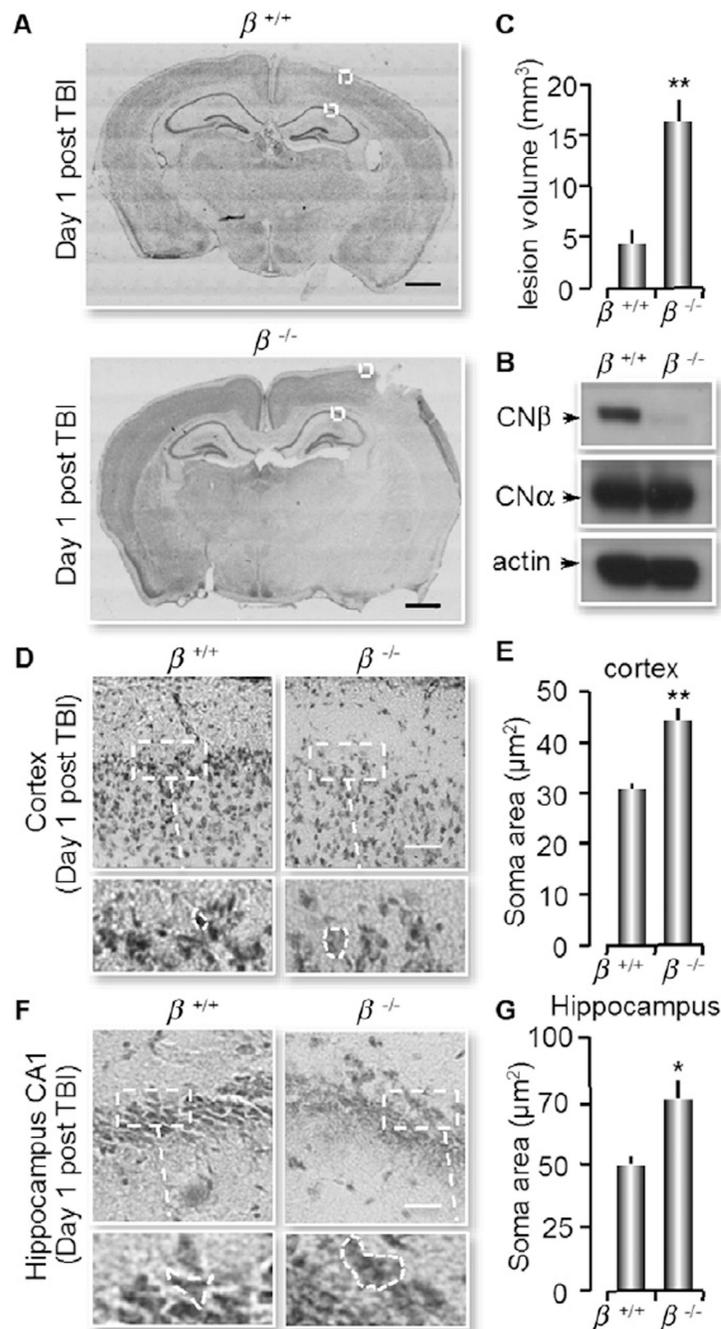
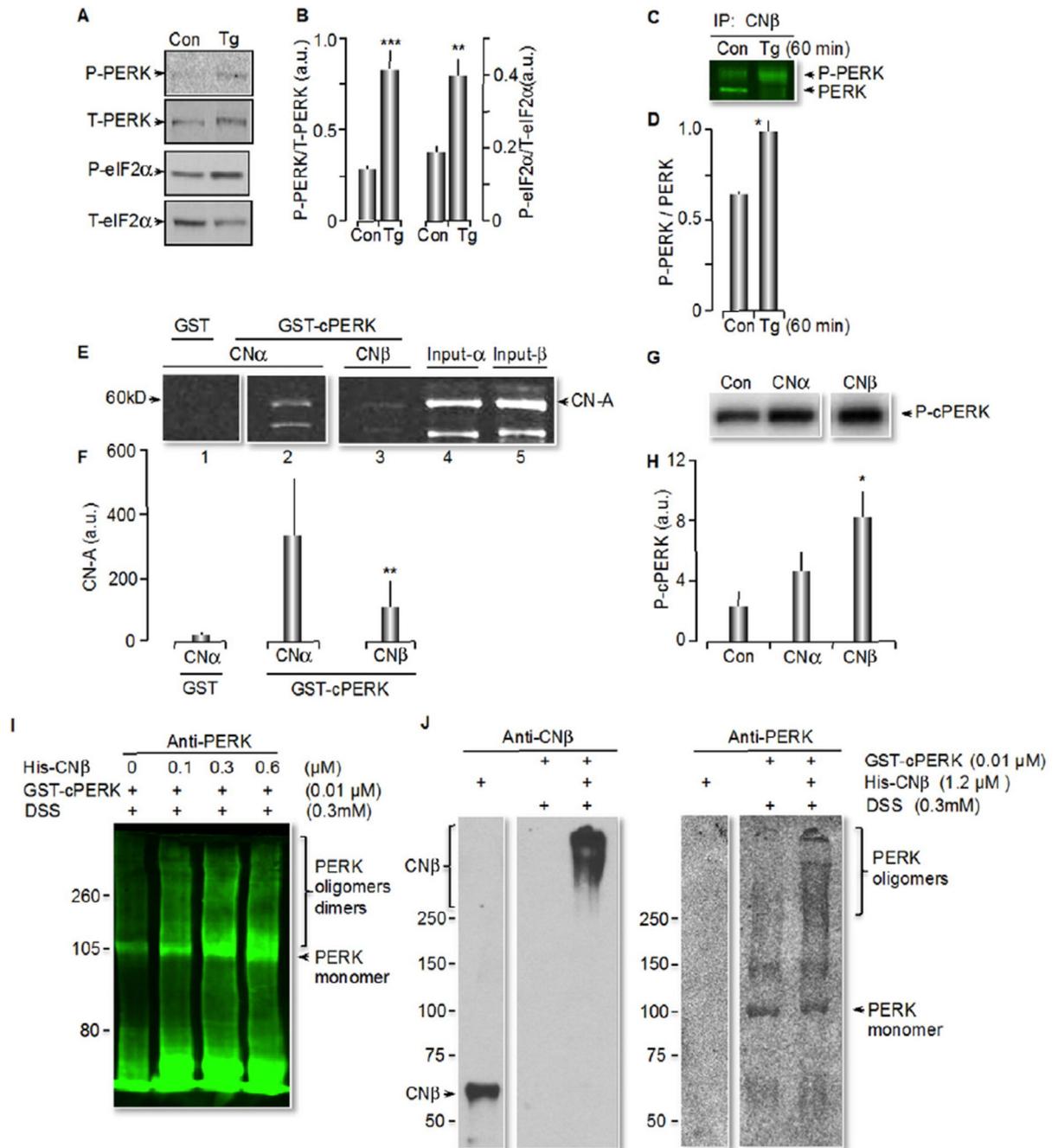


Fig. 2. CN β null mice exhibit higher lesion volume and increased cytotoxic edema in neurons after TBI. (A) Nissl staining of coronal sections from CN β wildtype ($\beta^{+/+}$) and null ($\beta^{-/-}$) mice 24 h post-TBI. Dashed boxes represent areas shown in D and F, respectively. Scale bar: 1 μ m. (B) Immunoblots of homogenized brain lysates from $\beta^{+/+}$ and $\beta^{-/-}$ mice for CN α and CN β . Actin was used as a loading control. (C) Quantification of lesion volume by Nissl staining shown in (A) ($n = 3$, mean \pm SEM, * $p < 0.05$, by unpaired two-tailed Student's t -test). (D, F) Higher magnification images from the cortex and the CA1 region of the

hippocampus from $\beta^{+/+}$ and $\beta^{-/-}$ mice. Scale bar: 10 μm . (E, G) Quantification of averaged neuron soma size in the cortex and the CA1 region of the hippocampus after TBI, respectively (n = 3, mean \pm SEM, * p < 0.05, ** p < 0.01 by unpaired two-tailed Student's t -test).

**Fig. 3.**

CN β physically interacts with PERK and promotes PERK phosphorylation and oligomerization. (A) C8D1A type I astrocytes were treated with vehicle (DMSO; Con) or 1 μ M of thapsigargin (Tg) for 1 h. Cell lysates were analyzed for total (T) and phosphorylated (P) PERK and eIF2 α using immunoblots. (B) Densitometry histograms after normalization to T-PERK or T-eIF2 α , respectively ($n = 3$, mean \pm SEM, ** $p < 0.01$ and *** $p < 0.001$ by unpaired two-tailed Student's t -test). (C) Primary mouse astrocytes were treated as indicated in (A) and then cross-linked with disuccinimidyl suberate (DSS) for 30 min.

Immunoprecipitation (IP) was performed with anti-CN β antibody and subsequent blots were probed with anti-PERK antibody. (D) Quantification of P-PERK/T-PERK densitometric ratio in (C) ($n = 3$, mean \pm SEM, $*p < 0.05$ by unpaired two-tailed Student's t test). (E) GST *pull-down* assay with either 8 nM of CN α or CN β . The proteins were incubated with glutathione sepharose 4B for 1 h, resolved on a 12% SDS-polyacrylamide gel and probed with an anti-calceurin PAN-A antibody. CN *pull-down* levels are shown for GST alone and GST-cPERK. (F) Densitometric histogram of (E) ($n = 3$, mean \pm SEM, $**p < 0.01$ by unpaired two-tailed Student's t test). (G) GST-cPERK was added to all reaction mixtures along with [$g^{32}P$] ATP in the absence or the presence of either of 0.043 mM of CN α or CN β . Reaction mixtures were run on SDS-PAGE and visualized by autoradiography. (H) Quantification of cPERK auto-phosphorylation density ($n = 5$, mean \pm SEM, $*p < 0.05$ by one-way ANOVA). (I) Recombinant His-CN β and GST-cPERK were incubated at the concentrations indicated, in the presence of 0.3 mM of DSS cross-linker for 30 min at room temperature. The ensuing protein complexes were run on SDS-PAGE and detected by immunoblotting using an anti-PERK antibody. (J) Recombinant His-CN β (1.2 mM) and GST-cPERK (0.01 mM) were incubated in the presence of 0.3 mM of DSS for 30 min at room temperature. The protein complexes were run on SDS-PAGE and detected by immunoblotting using antibodies against CN β and PERK.

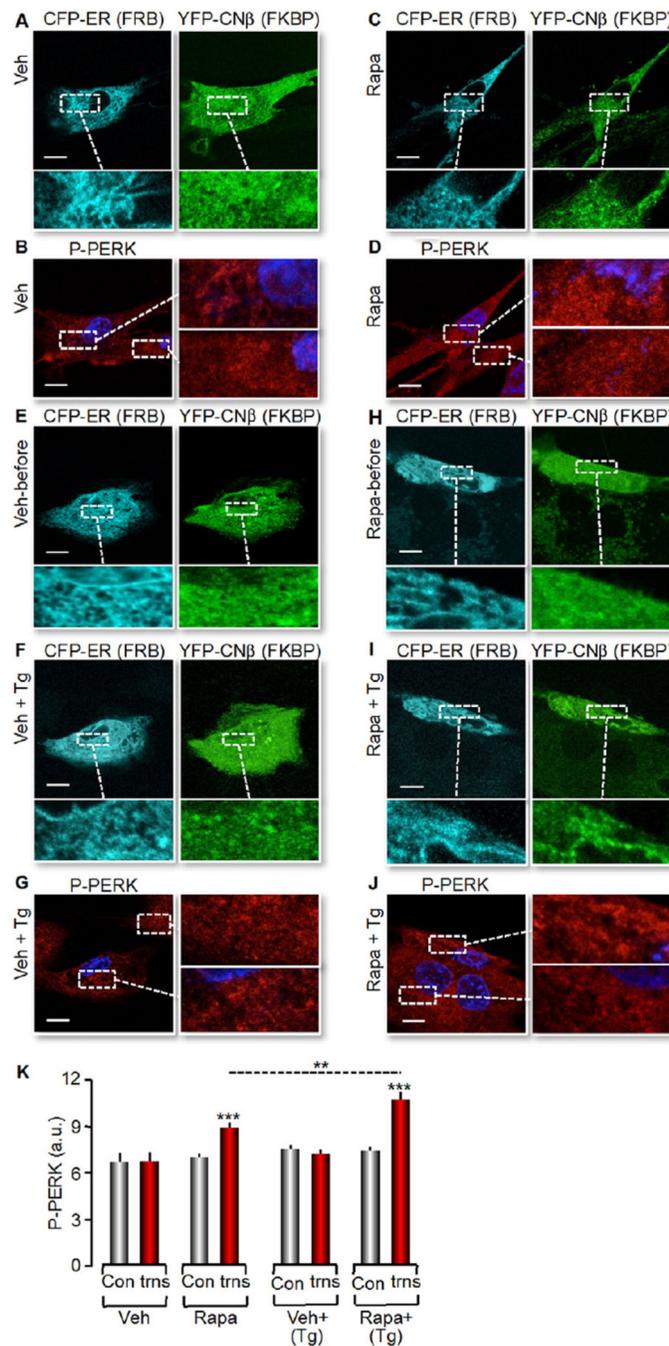


Fig. 4. Rapamycin induces CN β translocation to ER membrane and thereby promotes PERK phosphorylation. (A,C) Confocal images of human astrocytes expressing CFP-FRB-cytochrome 5 (cb5, ER anchor) and YFP-FKBP-CN β after addition of 100 nM of rapamycin (Rapa) for 30 min that induced translocation of the CN β construct to the ER. DMSO-treated cells were used as controls (Veh). (E-F, H-I) Confocal images of cells before and after addition of 100 nM of rapa for the first 10 min and then 25 nM Tg for an additional 20 min. DMSO and Tg treatments were used as controls (Veh + Tg). (B, D, G, J) Immunocyto-

chemistry was immediately performed to detect P-PERK (red). Nuclei were stained with DAPI (blue) in cells. Scale bar: 20 μm . (K) Quantification of fluorescence intensity of P-PERK in untransfected (Con) and transfected cells (trns) in (B, D, G, J) ($n > 20$ cells per group, mean \pm SEM, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA).

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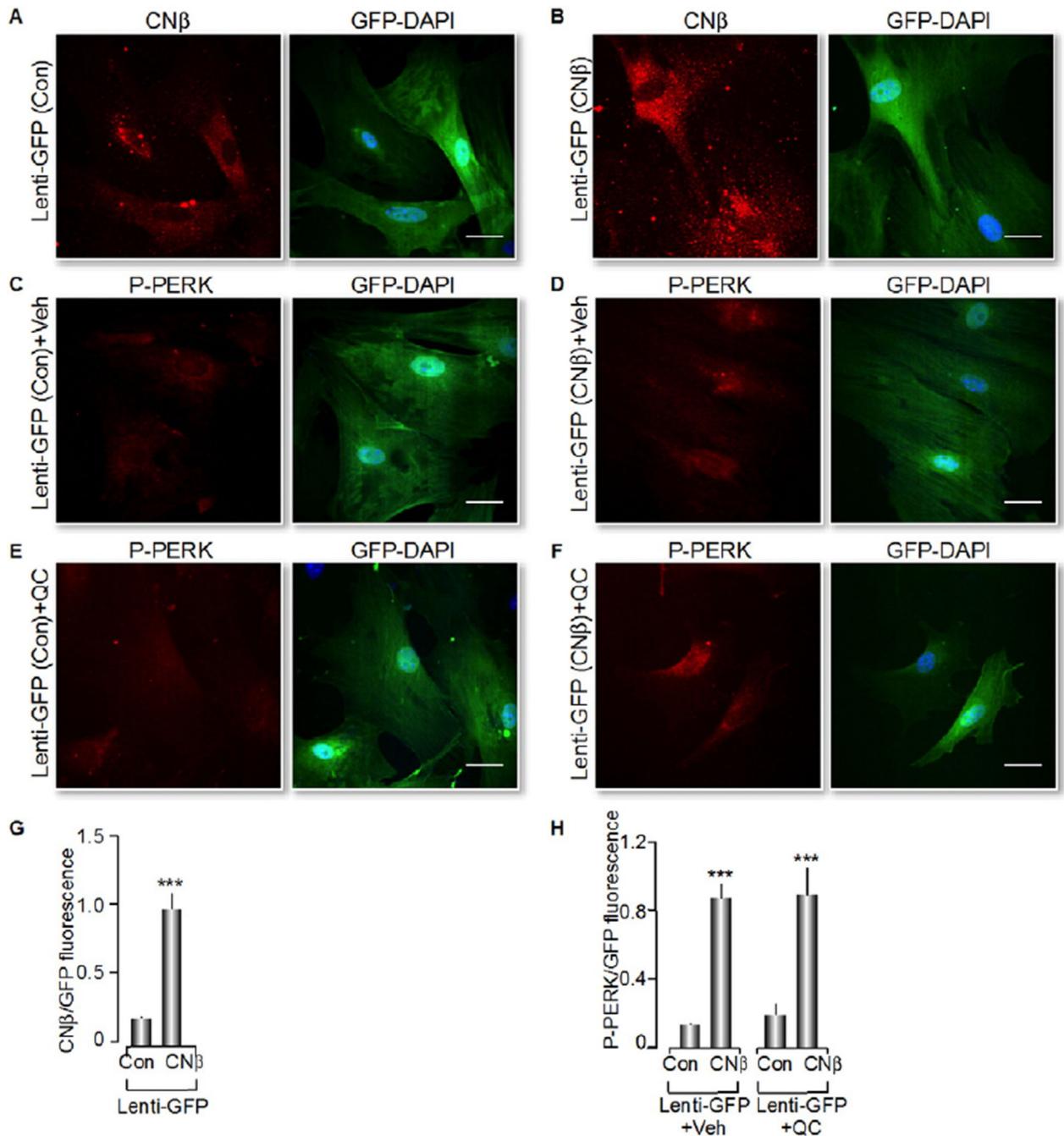


Fig. 5. CN β induces PERK phosphorylation independent of its phosphatase activity. (A, B) Primary astrocytes cultured from wildtype mice were transduced with pUltra lentivirus containing empty vector (Lenti-GFP Con) or Lenti-GFP (CN β) and stained for CN β (red). Nuclei were stained with DAPI (blue). Presence of virus is indicated by GFP (green). (C–F) Infected astrocytes were exposed to DMSO (Veh) or 40 μ M of Quercetin (QC) for 20 min. Immunocytochemistry was carried out for P-PERK (red) and DAPI (blue). Images were sequentially acquired. Scale bar: 20 μ m. (G) Ratio of fluorescence intensity (CN β /GFP) in (A, B) from

three independent experiments in which at least 10 cells were quantified per experiment (mean \pm SEM, *** $p < 0.001$ by unpaired two-tailed Student's t -test). (H) Ratio of fluorescence intensity (P-PERK/GFP) in (C–F) from three independent experiments in which at least 10 cells were quantified per experiment (mean \pm SEM, *** $p < 0.001$ by one-way ANOVA).

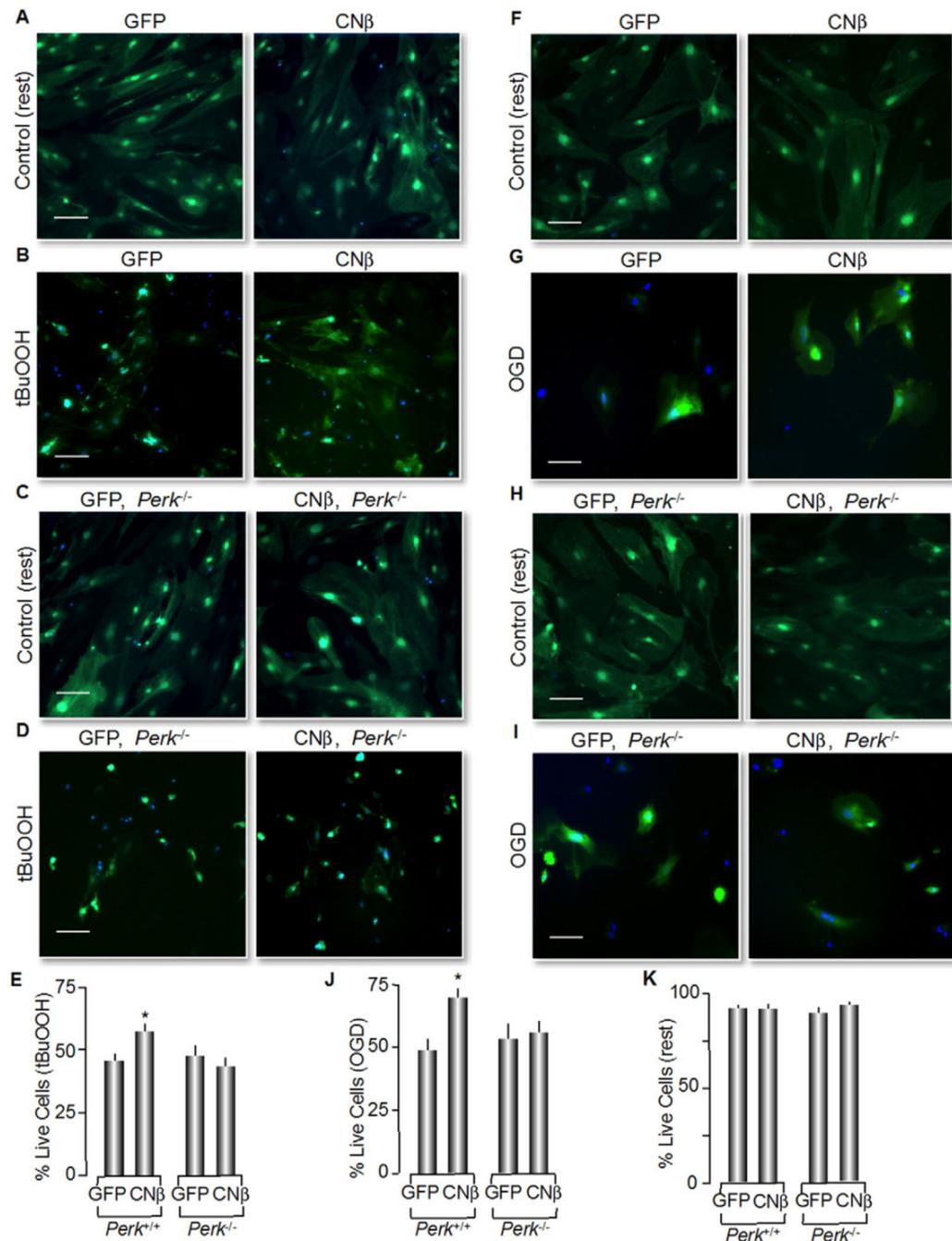


Fig. 6. Lentiviral expression of CN β increases cell viability of wildtype but not PERK-null astrocytes. (A, C, F, H) Astrocytes cultured from *Perk*^{+/+} and *Perk*^{-/-} mice were infected with lentivirus pUltra lenti-GFP or pUltra lenti-GFP (CN β) (green). Cells in (B, D) were exposed to tBuOOH for 2 h and cells in (G, I) were exposed to OGD for 1 h. Cells in (K) were at rest. Nuclei were stained using Hoechst 33342 (blue). Cells with GFP expression were viable cells. Scale bar: 80 μ m. (E, J) Quantification of percent live cells in lentivirus-

infected astrocytes in (B, D, G, I) from three independent experiments in which 5 random fields were counted (mean \pm SEM, * $p < 0.05$ by one-way ANOVA).

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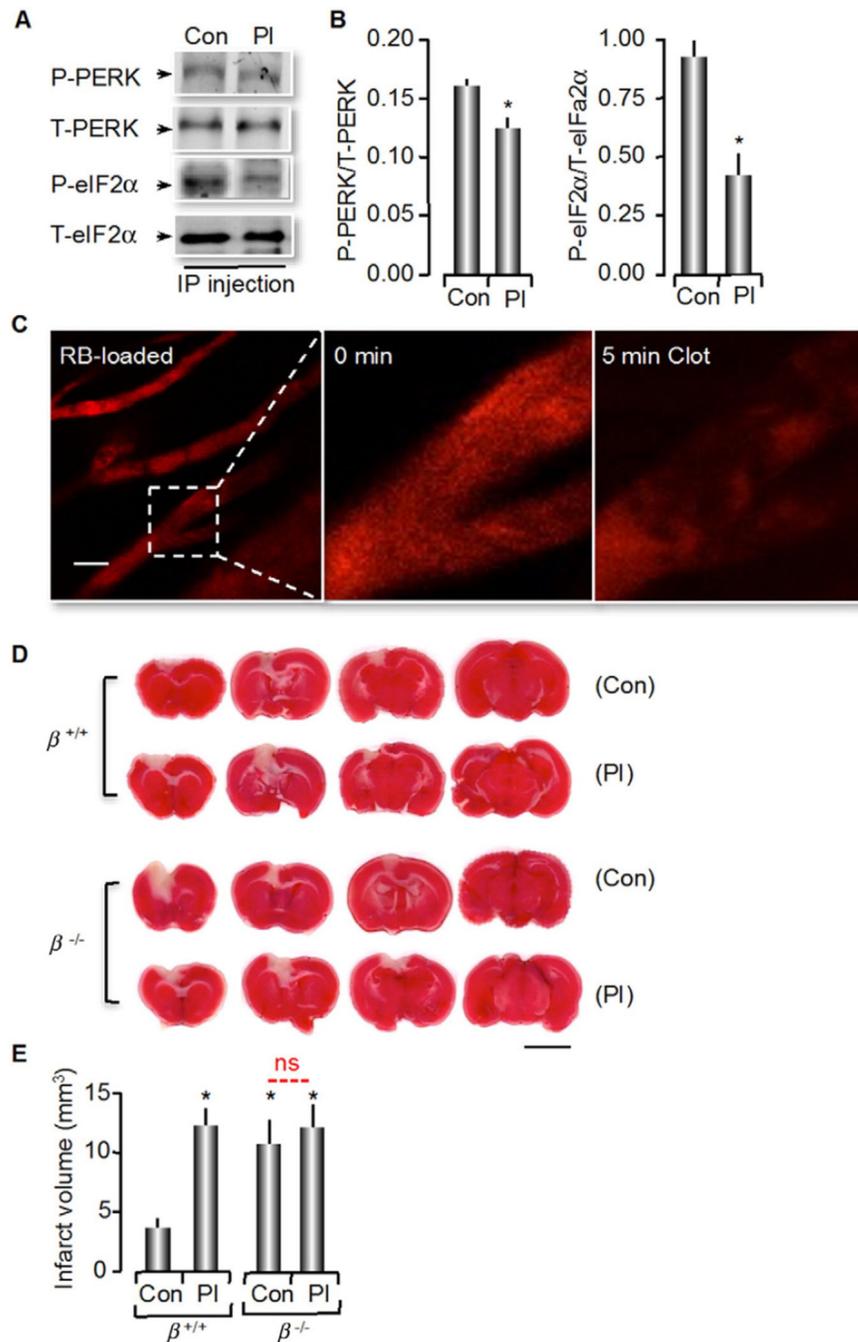


Fig. 7. CN β null mice exhibit larger infarct size after photo thrombotic stroke as compared to CN β null mice with inhibition of PERK activity. PERK inhibitor (PI) or DMSO (Con) was intraperitoneally injected into CN β wildtype ($\beta^{+/+}$) and null ($\beta^{-/-}$) mice 12 h before stroke. (A) Homogenized brain lysates of mice at 12 h post-injections were analyzed for T-PERK, P-PERK, T-eIF2 α and P-eIF2 α by immunoblots. (B) Quantification of immunoblots in (A) ($n = 3$, mean \pm SEM, * $p < 0.05$ by unpaired two-tailed Student's t -test). (C) Rose bengal (RB) dye-induced photothrombotic model. Confocal images show that blood vessel lumens

were filled with RB dye. Region marked by dashed box was irradiated with 543 nm laser light. After approximately 5 min, a thrombotic clot was formed with the absence of blood flow. Scale bar: 20 μm . (D) Tetrazolium chloride (TTC) staining of serial coronal sections 24 h post-photothrombotic stroke on $\beta^{+/+}$ and $\beta^{-/-}$ mice with DMSO (Con) or PI injection. Scale bar: 5 mm. (E) Quantification of infarct volume 24 h post-stroke in (D) ($n = 5$, mean \pm SEM, $*p < 0.05$ by one-way ANOVA).

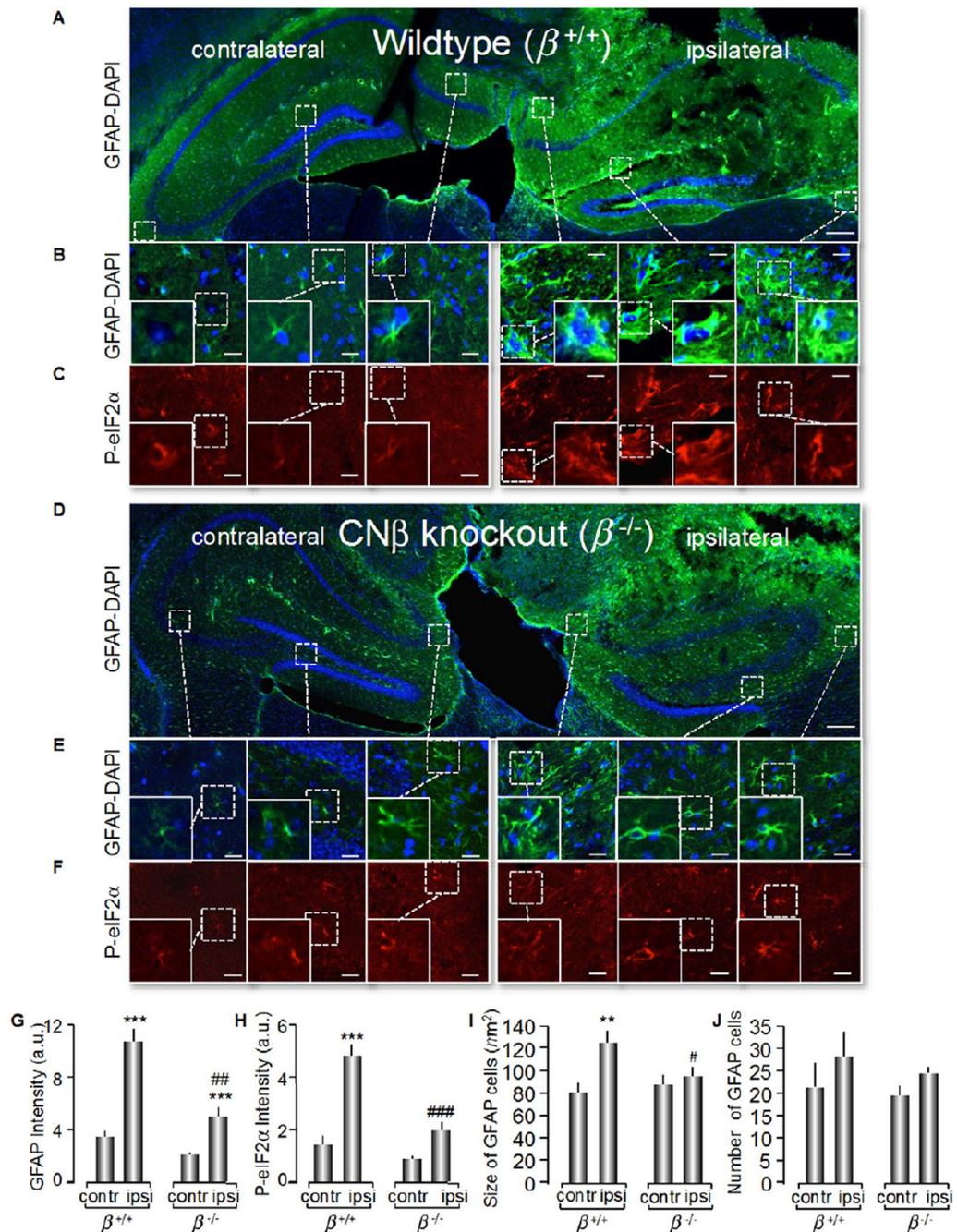


Fig. 8. β^{+/+}, not β^{-/-} mice, exhibit enlarged astrocytes as well as up-regulated P-eIF2α in reactive astrocytes after stroke. Mouse brains harvested at post 24 h of stroke were sectioned and stained with antibodies against P-eIF2α (red), GFAP (green) and DAPI (nuclei, blue). Montage images were acquired on a Zeiss LMS 710 confocal microscopy with a Nikon 20× objective. (A, D) The β^{+/+} and β^{-/-} brain sections with GFAP and DAPI staining. (B, E) The high magnification of images from the dashed boxes in A and D, respectively. (C, F) The brain sections from B and E in P-eIF2α staining, respectively. (G–J) Quantification of

fluorescence intensities of GFAP and P-eIF2 α in astrocytes, the size and the number of GFAP positive astrocytes (n = 3, four random regions analyzed from each side, mean \pm SEM). ** $p < 0.01$ and *** $p < 0.001$, compared with the contralateral(contr)side; # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$, compared with the ipsilateral (ipsi) side of $\beta^{+/+}$ respectively, by 1-way ANOVA. Scale bar: 250 μm (A, D); 20 μm (B, C, E, F).