Corrections

NEUROSCIENCE

The authors note that Fig. 4 appeared incorrectly. In panel 4A, the wrong image for the β-actin Western blot was placed in the figure. The corrected figure and its legend appear below. This error does not affect the conclusions of the article.

Fig. 4. Silencing of eIF4G1 reduces protein synthesis in neurons and exacerbated OGD-induced death. (A) Neurons were transfected with either shRNA lentivirus targeting rat eIF4G1 (sh4G) or a nontargeting shRNA virus (nt) for 72 and 96 h. Cell lysates were analyzed by Western blot and probed to determine eIF4G1 protein level. eIF4G1 silencing was effective, but other translation initiation factors were not altered. (B) After 1 h OGD and 24 h reperfusion, protein synthesis was reduced in control (nt) and more so in sh4G cultures. Cotransfection of neurons for 72 h with human HA-eIF4G1 and sh4G (4G+sh4G) significantly restored protein synthesis relative to sh4G under control conditions and after 1 h OGD and 24 h reperfusion. (C) Viability was greater in 4G+sh4G cotransfected neurons compared with control (nt) and sh4G transfected neurons following OGD; the data parallel the changes in protein synthesis shown in B under the same conditions. (D) Western blot showed eIF4G1 silencing in sh4G transfected neurons and expression at greater than control levels in 4G+sh4G transfected neurons under control conditions (nt) and after 1 h OGD and 24 h reperfusion with little change in other initiation factors. Bars represent mean ± SD of n = 3–4 independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001.

www.pnas.org/cgi/doi/10.1073/pnas.1202401109

NEUROSCIENCE, PSYCHOLOGICAL AND COGNITIVE SCIENCES

The authors note that on page 2126, left column, lines 6–10 of the abstract, “Behaviorally, monkeys discriminated between cues signaling small and large rewards, and between cues signaling rewards to self only and reward to both self and another monkey, with a preference for the former over the latter in both instances,” should instead appear as “Behaviorally, monkeys discriminated between cues signaling large and small rewards, and between cues signaling rewards to self only and reward to both self and another monkey, with a preference for the former over the latter in both instances.”

www.pnas.org/cgi/doi/10.1073/pnas.1201821109

EARTH, ATMOSPHERIC, AND PLANETARY SCIENCES

The authors wish to note the following: “We would like to draw attention to the work of Molinero and co-workers (1, 2), which is relevant for our article. Moore and Molinero (2) very recently conducted a large scale molecular dynamics study into the structure of ice that crystallizes at 180 K. They find that the ice that forms in their simulations contains both cubic and hexagonal sequences with a ratio of about 2:1, similar to their previous study on ice formation in a nanopore (1). This work supports our conclusion that the material referred to as cubic ice in the literature is actually stacking disordered ice, but our experiments and simulations indicate that the ratio of cubic to hexagonal sequences is not restricted to 2:1.”


www.pnas.org/cgi/doi/10.1073/pnas.1201020109

www.pnas.org/cgi/doi/10.1073/pnas.1201821109
AGRICULTURAL SCIENCES


The authors note that a reference to an article by De Oliveira et al. was omitted. The complete reference appears below.

As a result of this omission, Fig. 3 appeared incorrectly. The corrected figure and its legend appear below. These errors do not affect the conclusions of the article.


Fig. 3. Maize grain yield plotted against fossil-fuel energy inputs (A) and GWP (B). Lines indicate average 3-y median (solid line) and fifth and 95th percentiles (dashed lines) for net NER and GWPi calculated for irrigated maize in Tri-Basin NRD. Published data for other US maize systems are shown for comparison (open symbols; Table S1). (C) Relationship between GWPi and net energy yield for irrigated maize in Tri-Basin NRD.

www.pnas.org/cgi/doi/10.1073/pnas.1201296109
Corrections

**NEUROSCIENCE**


The authors note that Fig. 3 appeared incorrectly. In panel 3E, the wrong image for the β-actin loading control was used for the Western blot. The corrected figure and its legend appear below. This error does not affect the conclusions of the article.

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**Fig. 3.** Neuroprotection by eIF4G1 following ischemia is at least partially protein synthesis dependent. (A) Immunostaining for the HA tag was performed to assess transfection efficacy of lentiviral-mediated overexpression of HA-eIF4G1, and neuronal colocalization was demonstrated by costaining for MAP2. (B) Levels of initiation factors in primary neurons overexpressing HA-eIF4G1 (4G) compared with those in neurons expressing GFP are shown by Western blot. (C and D) Control (GFP-transfected) neurons and 4G-transfected neurons were untreated or exposed to 50 μM CHX for 24 h or subjected to 1 h OGD and 24 h reperfusion without or with 50 μMCHX. After 24 h, treated neurons were assessed for protein synthesis (cpm per microgram protein) (C) and viability (D). Both protein synthesis and survival were increased by 4G overexpression. (E) Western blot analysis showing the effect of GFP- or eIF4G1-transfection on the expression of regulatory translation initiation factors by neurons without OGD or subjected to 1 h OGD at 0, 2, and 24 h reperfusion. Specifically, P-eIF2α was transiently increased at 0 and 2 h as in Fig. 1F, and eIF4G1 was decreased at 24 h in control neurons. Bars represent mean ± SD of *n* = 3–4 independent experiments. *P* < 0.05, **P** < 0.01, and ***P*** < 0.001 compared with control and where indicated, **P** < 0.01 compared with control, GFP-transfected, CHX-treated neurons.

www.pnas.org/cgi/doi/10.1073/pnas.1320015110
PLANT BIOLOGY

The authors note that Table 1 appeared incorrectly. The corrected table appears below. This error does not affect the conclusions of the article.

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Table 1. TALE RVDs

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DEVELOPMENTAL BIOLOGY

The authors note that in the Acknowledgments lines 1–6, “We thank Prof. Noboru Mizushima (Tokyo Medical and Dental University) and Prof. Masaru Okabe (Osaka University) for providing the GFP-LC3#53 and B6D2-Tg(CAG/Su9-DsRed2, Acr3-EGFP)RBGS002Os transgenic mice, respectively; and Profs. Shaorong Gao (National Institute of Biological Sciences) and Wei Li (Institute of Zoology, Chinese Academy of Sciences) for providing the Zp3-Cre and Atg5fl/fox mice, respectively.” should instead appear as “We thank Prof. Noboru Mizushima (Tokyo Medical and Dental University) and Prof. Masaru Okabe (Osaka University) for providing the GFP-LC3#53 and B6D2-Tg(CAG/Su9-DsRed2,Acr3-EGFP)RBGS002Os transgenic mice, respectively. Atg5fl/fox mice were obtained from RIKEN Bioresource Center in Japan [Hara et al. (2006) Nature, 441, 885—889] and Zp3-Cre mice were from Jackson Lab.”

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BIOPHYSICS AND COMPUTATIONAL BIOLOGY

The authors note that the author name Feng C. Tsai should instead appear as Feng-Ching Tsai. The corrected author line appears below. The online version has been corrected.

Kevin Carvalho, Feng-Ching Tsai, Edouard Lees, Raphaël Voituriez, Gijsje H. Koenderink, and Cécile Sykes

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Ischemia-induced calpain activation causes eukaryotic (translation) initiation factor 4G1 (eIF4G1) degradation, protein synthesis inhibition, and neuronal death

Peter S. Vosler, Yanqin Gao, Christopher S. Brennan, Akiko Yanagiya, Yu Gan, Guodong Cao, Feng Zhang, Simon J. Morley, Nahum Sonenberg, Michael V. L. Bennett, and Jun Chen

Persistent protein synthesis inhibition (PSI) is a robust predictor of eventual neuronal death following cerebral ischemia. We thus tested the hypothesis that persistent PSI inhibition and neuronal death are causally linked. Neuronal viability strongly correlated with both protein synthesis and levels of eukaryotic (translation) initiation factor 4G1 (eIF4G1). We determined that in vitro ischemia activated calpain, which degraded eIF4G1. Overexpression of the calpain inhibitor calpastatin or eIF4G1 resulted in increased protein synthesis and increased neuronal viability compared with controls. The neuroprotective effect of eIF4G1 overexpression was due to restoration of cap-dependent protein synthesis, as well as protein synthesis-independent mechanisms, as inhibition of protein synthesis with cycloheximide did not completely prevent the protective effect of eIF4G1 overexpression. In contrast, shRNA-mediated silencing of eIF4G1 exacerbated ischemia-induced neuronal injury, suggesting eIF4G1 is necessary for maintenance of neuronal viability. Finally, calpain inhibition following global ischemia in vivo blocked decreases in eIF4G1, facilitated protein synthesis, and increased neuronal viability in ischemia-vulnerable hippocampal CA1 neurons. Collectively, these data demonstrate that calpain-mediated degradation of a translation initiation factor, eIF4G1, is a cause of both persistent PSI and neuronal death.

Global ischemia | eukaryotic (translation) initiation factor 4G1 cleavage

Protein synthesis is a highly regulated process essential for normal growth, development, and maintenance of cells. Various cellular stressors including viral infection, nutrient deprivation, and heat shock inhibit protein synthesis (1). The characteristics of the stress are important in the eventual recovery of protein synthesis. A sublethal insult such as hypoglycemia initially inhibits protein synthesis, but translation returns upon nutrient restoration. In contrast, inhibition of protein synthesis due to infection or other stimuli can be irreversible and associated with cell death (2).

Stimuli inducing programmed cell death have been repeatedly shown to inhibit protein synthesis (2). Likewise, agents that inhibit protein synthesis induce cell death (3, 4). However, in no case have protein synthesis inhibition and cell death been directly linked. The relationship between persistent protein synthesis inhibition (PSI) and cell death is exemplified by neurons following an ischemic insult (5), and induction of ischemia provides a useful model to study the mechanisms leading to PSI and cell death.

PSI occurs in all neurons destined to die following global ischemia (5). In contrast to the rapid restoration of energy metabolism (6), recovery of protein synthesis is slower and may fail in ischemia-susceptible brain regions (7, 8). This is also demonstrated by focal ischemia models in which the brain regions where protein synthesis is inhibited following 1 h of ischemia predict the infarct volume at 3 d (9).

Ischemia (5) and other stimuli inducing programmed cell death (2) cause polysome disassociation, indicating inhibition of translation initiation. Examination of the levels and phosphorylation states of a number of critical translation initiation factors following cerebral ischemia suggests that depressed levels of the scaffolding protein responsible for delivering the mRNA to the ribosome for translation, eukaryotic initiation factor 4G1 (eIF4G1), are correlated with persistent PSI (10). Excitotoxicity caused by ischemia and neurodegenerative diseases is known to result in NMDA-induced calcium influx into neurons, resulting in activation of the protease calpain (11). Calpain is known to degrade eIF4G1 in vitro (12), and it has been implicated in ischemia-induced decreases in eIF4G1 (13); inhibition of calpain also provides neuroprotection in a focal ischemia model (14).

The purpose of the present study was to determine the underlying mechanisms mediating persistent PSI and to determine whether persistent PSI is directly related to neuronal death in both in vitro and in vivo ischemia models. Our results indicate that eIF4G1 is degraded following both in vitro and in vivo ischemia in a calpain-dependent manner. Moreover, we demonstrate that maintenance of near normal levels of a translation initiation factor, eIF4G1, either by overexpression or calpain inhibition results in decreased PSI and increased neuroprotection against oxygen–glucose deprivation (OGD) in vitro and global ischemia in vivo.

Results

PSI and Viability Are Correlated with Duration of Ischemia. We initially sought to establish our pure (~97%) primary neuronal culture coupled with OGD as a viable model of persistent PSI observed following in vivo ischemia. To model both ischemia-sensitive and -resistant areas of the brain, we determined durations of OGD that were sublethal, moderately lethal (i.e., lethal to a fraction of the cultured cells), and severely lethal (i.e., lethal to most of the cells). Viability was assayed at 24 h after 0.5, 1, and 1.5 h OGD by Alamar Blue reduction (metabolic state), lactate dehydrogenase (LDH) release (membrane breakdown), and nuclear fragmentation (apoptosis). Sublethal (0.5 h) OGD did not result in significant loss of viability as determined by any of these assays. However, viability measured by Alamar Blue fluorescence after OGD for 1 h and 1.5 h decreased by 50% and...
On the basis of the above observations, we examined the effects of OGD on protein synthesis levels with quantitation following 1 h OGD shows a progressive decrease of eIF4G1 to 50% of control during 2–24 h reperfusion. This initial evidence supports our hypothesis that a decrease in the levels of eIF4G1 correlates with decreased viability and PSI at 24 h reperfusion after 1 h and 1.5 h OGD, respectively. Thus, viability was positively correlated with protein synthesis levels.

Levels of eIF4G1 Correlate with Persistent PSI. Continuing to validate our in vitro model of ischemia, we compared changes in in initiation factor levels and phosphorylation states induced by in vitro OGD to the results in the in vivo literature. As seen in vivo (15–18), there were no changes in many of the regulatory initiation factors following 1 h OGD (Fig. 1E and Fig. S1 D–F). Lethal (1 h) but not sublethal OGD, however, resulted in the progressive decrease of eIF4G1 to ∼50% of control during 2–24 h reperfusion (Fig. 1C and D). Notably, the decrease in the level of eIF4G1 correlates with decreased viability and PSI at 24 h reperfusion. This initial evidence supports our hypothesis that loss of critical levels of eIF4G1 is responsible for PSI following ischemia and ultimately cell death. Of note, total levels of 4EBP did decrease at 6–24 h, but because 4EBP is a negative regulator of translation, decreased 4EBP is not expected to contribute to PSI.

Phosphorylation of eIF2α (P-eIF2α) is a mechanism known to inhibit translation by blocking delivery of tRNAs to the ribosome (1). Transient increases in P-eIF2α with levels returning to baseline by 6 h of reperfusion have been repeatedly observed in vivo ischemia (5). OGD (1 h) doubled the amount of P-eIF2α, but the level returned to baseline within 2 h of reperfusion (Fig. 1 F and G). These data validate our in vitro model as an adequate representation of in vivo ischemia. Furthermore, the onset and duration of the increase in P-eIF2α indicate its potential involvement in initial PSI, but the transience of the phosphorylation and reversion in vivo evidence demonstrating that blocking P-eIF2α has no effect on PSI (10) exclude its role in mediating persistent PSI.

Calpain Is Activated Following In Vitro Ischemia. Neuronal death following OGD has been linked to both caspase- and calpain-dependent mechanisms (19, 20), and eIF4G1 has been shown to be a substrate of both caspase-3 (3) and calpain (12, 13). We thus attempted to determine whether these proteases are involved in the decrease of eIF4G1 following OGD.

We first evaluated the cleavage products of the caspase-3 and calpain substrate α-spectrin following OGD. Both proteases cleave the 240-kDa protein, leaving a 150-kDa fragment, but calpain further cleaves it to a 145-kDa fragment whereas caspase-3 produces a 120-kDa fragment. Following 1 h OGD there was a decrease in the full-length protein and a corresponding increase in the appearance of the calpain-induced 145-kDa fragment, although there was no change in the caspase-induced 120-kDa fragment (Fig. S2 A and B). This suggests that calpain rather than caspase was activated in our model, which was confirmed with caspase and calpain activity assays and by inhibition of caspases following 1 h OGD. Caspase activity did not increase above control, and caspase inhibition failed to provide neuroprotection following OGD (Fig. S2 C, E, and F). In contrast, calpain activity increased immediately after OGD and for 6 h of reperfusion (Fig. S2D). These findings are in agreement with previous studies of OGD in mixed glial and neuronal cultures (20).

Calpain Inhibition Blocks eIF4G1 Cleavage, Reduces PSI, and Increases Survival. On the basis of the above observations, we examined the role of calpain on eIF4G1 levels, protein synthesis, and viability after OGD. Calpain inhibition was achieved by overexpression of calpastatin (Cps), the endogenous inhibitor of calpain (11), with an HA tag (Fig. 2 A and B). Calpastatin overexpression increased full-length eIF4G1 and decreased the 145-kDa band of α-spectrin following 1 h OGD and 24 h reperfusion. Treatment with ZVAD did not alter the α-spectrin cleavage profile (Fig. S3 A and B). Overexpression of calpastatin, but not inhibition of caspases, resulted in significantly increased levels of eIF4G1 after 1 h OGD and 24 h reperfusion compared with GFP-transfected controls (Fig. 2 B and C). Analysis of protein synthesis following 1 h OGD in calpastatin and GFP-transfected neurons showed an initial inhibition of protein synthesis with increased recovery in the calpastatin-overexpressing neurons compared with time-matched GFP controls at 12 and 24 h reperfusion (Fig. 2D). The increase in protein synthesis was associated with increases in viability at 24 h reperfusion (Fig. 2E and Fig. S3C). This result provides further evidence that calpain is mediating eIF4G1 cleavage following OGD in cultured neurons and that decreases in eIF4G1 account for persistent PSI and, ultimately, neuronal death following ischemia.

eIF4G1 Is a Calpain Substrate. To confirm that eIF4G1 is a substrate of calpain (12, 13), we used a cell-free assay where we mixed recombinant eIF4G1 with recombinant calpain I and activated the enzyme with calcium. Calpain I clearly cleaved eIF4G1, incubated with both the endogenous calpain inhibitor calpastatin and the pharmacological calpain-specific inhibitor ALLN abrogated eIF4G1 cleavage (Fig. S4A).

We next examined the time course of eIF4G1 cleavage by calpain. Incubation of recombinant eIF4G1 with calpain for specified times resulted in progressive cleavage of eIF4G1 into size-specific products as shown by probing with eIF4G1 N- and
C-terminal directed antibodies (Fig. S4 B and C). Notably, ~50- and 66-kDa fragments were seen using the C-terminal antibody, which corroborates the findings in neuronal cultures following OGD (Fig. S4F) and in brain following focal ischemia (18).

In a further attempt to identify the cleavage sites of calpain on eIF4G1, we used recombinant protein fragments of eIF4G1. Recombinant calpain cleaved eIF4G1 fragments consisting of amino acids 197–674 and 675–1129 (Fig. S4E). Analysis of the cleavage products by mass spectrometry positively identified the bands as eIF4G1 and confirmed the molecular weights as 66, 50, and 16 kDa (Fig. S4D). Unfortunately, multiple attempts to identify the specific sequence targeted by calpain using Edman degradation failed. Together, these results suggest that calpain does not recognize a single site on eIF4G1, but progressively cleaves the protein to either modify or abolish its function.

**Overexpression of eIF4G1 Is Neuroprotective.** We hypothesized that neuronal death following ischemia is directly related to failure to preserve levels of eIF4G1. To test this hypothesis, we overexpressed eIF4G1 in primary neurons using a lentiviral vector. The lentivirus expressing human HA-eIF4G1 was present in ~75–90% of neurons (Fig. 3A). Transfection of eIF4G1 did not affect the level of several other initiation factors or the phosphorylation state of eIF2α (Fig. 3B). Overexpression of eIF4G1 resulted in increased protein synthesis and neuronal viability 24 h after 1 h OGD compared with GFP control (Fig. 3 C and D). To determine whether the neuroprotective effect of eIF4G1 was directly due to protein synthesis, we inhibited protein synthesis using cycloheximide (CHX). A concentration response curve of CHX application demonstrates that concentrations of CHX that substantially inhibited protein synthesis for 24 h also resulted in neuronal death (Fig. S5A). Protein synthesis was inhibited to ~30% of untreated levels following 24 h of exposure to CHX (50 μM) in both groups under control conditions and was nearly completely inhibited in both groups after OGD (Fig. 3C).

Examination of neuronal viability revealed that concentrations of CHX used to inhibit protein synthesis were mildly but significantly neurotoxic, causing ~40% of neurons to die (Fig. 3D), which corroborates previous findings in primary neurons (4). The importance of protein synthesis in maintenance of viability following in vitro ischemia was further substantiated by the observation that CHX treatment significantly decreased neuronal viability following OGD in both the GFP- and eIF4G1-overexpressing cultures. Significantly reduced viability was also observed when comparing control cultures treated with CHX to OGD- and CHX-treated cultures (Fig. 3D, third and seventh bars). Importantly, inhibition of protein synthesis with CHX only partially blocked the neuroprotective effect of eIF4G1, as eIF4G1-overexpressing cultures treated with CHX had increased viability compared with OGD- and CHX-treated GFP neurons (Fig. 3D, seventh and eighth bars). This suggests that overexpression of eIF4G1 may confer neuroprotection via mechanisms other than by increasing global translation.

To further support our findings that persistent PSI is pathological, we examined the effect of eIF4G1 overexpression on the time course of protein synthesis following 1 h OGD. As seen with control cultures and calpastatin-overexpressing neurons (Fig. 2D), protein synthesis was immediately inhibited following 1 h OGD in both control and eIF4G1-overexpressing neurons (Fig. S5B). However, protein synthesis began to recover at 12 h reperfusion in eIF4G1-overexpressing neurons but not in controls.

Examination of protein levels following OGD revealed greater eIF4G1 levels at 2 and 24 h following OGD in eIF4G1- compared with GFP-overexpressing neurons (Fig. 3E and Fig. S5C). Furthermore, OGD resulted in a similar P-eIF2α profile in GFP- and eIF4G1-overexpressing neurons (Figs. 3E and 1E). Again, this suggests that P-eIF2α does not contribute to persistent PSI or delayed neuronal death. Instead, the data collectively support the inference that maintenance of critical levels of eIF4G1 is
essential for neuronal survival following OGD and that the
neuroprotection may be in part translation independent.

shRNA Silencing of eIF4G1 Exacerbates OGD-Induced Death. Our
overexpression experiments demonstrate that maintenance or
increase of eIF4G1 levels following OGD can restore protein
synthesis and provide neuroprotection. The next step was to
determine whether reducing the eIF4G1 level was sufficient to
cause neuronal death. To determine this we transfected a lentiviral
vector with shRNA targeting rat eIF4G1 into our primary
cultures. Knockdown of eIF4G1 to ~20% of control values was
observed at 72 h and 96 h posttransfection without affecting
levels of other initiation factors tested (Fig. 4A and Fig. S6A).
Knockdown of eIF4G1 96 h posttransfection resulted in a 24% decrease in protein synthesis and a 17% decrease in neuronal
viability relative to a control, non-targeting (nt) shRNA (Fig. 4 B
and C). The decreases in translation are similar to the effects of
eIF4G1 silencing observed in a recent study using a breast cancer
cell line, and the incomplete loss of protein synthesis may be due to
the presence of the eIF4G1 homologs eIFG2 and p97 (21). Using HeLa cell extracts and adding purified calpain, we found that all three proteins are equally vulnerable to cleavage by
calpain (Fig. S7), and overexpression of eIF4G1 alone was suf-
ficient to increase protein synthesis and provide neuroprotection
following ischemia (Fig. 3 C and D). Ischemia-induced decreases in
cap-dependent translation and viability were exacerbated by knockdown
of eIF4G1 (Fig. 4 B and C). To determine whether the exacer-
bation of injury was related to loss of eIF4G1, we overexpressed
human eIF4G1 in neurons depleted of endogenous eIF4G1; this
treatment increased protein synthesis and neuronal viability
(Fig. 4 B and C, fifth and sixth bars). Recovery of protein syn-
thesis and viability was again associated with greater eIF4G1
levels, and there were no changes in levels of several other
translation initiation factors (Fig. 4D and Fig. S6B).

Cap-Dependent Translation Is Inhibited Following OGD. Cap-de-
pendent translation via translation initiation factor binding of the
7-methyl-guanosine cap is the predominant means for endoge-

ous cellular mRNA translation (1). We thus wanted to de-
terminewhether cleavage of eIF4G1 by calpain following OGD
would affect cap-dependent translation following OGD. The
sole cap-dependent cellular β-globin 5′-UTR was inserted into
Renilla luciferase expression vectors (SI Materials and Methods)
and transfected into the neuronal cultures for 2 h at the times
indicated. The viral encephalomyocarditis virus (EMCV) 5′-UTR
was also inserted upstream of a Firefly luciferase vector and
transfected into all cells as a transfection control. Following
incubation, the cells were lysed and luciferase activities were
measured.

Under control conditions, the capped β-globin was robustly
translated (Fig. S8A). Following OGD, β-globin translation was
severely inhibited at all times measured (3, 7, and 25 h). We also
wanted to determine whether the restoration of protein synthesis
due to eIF4G1 overexpression was due to reinstatement of cap-
dependent translation. As in the previous experiment, capped
β-globin mRNA was transfected into eIF4G1- and GFP-trans-

fected neurons at the indicated times after OGD. Luciferase
activity indicating cap-dependent translation was inhibited fol-
lowing OGD in GFP-transfected neurons for at least 24 h (Fig.
S8B). Overexpression of eIF4G1, however, increased cap-de-
pendent translation to near control levels by 25 h after the insult.
These results indicate that the increase in protein synthesis
caused by increasing eIF4G1 levels was, at least in part, due to
increased cap-dependent translation.

Calpain Inhibition Reduces Ischemia-Induced Persistent PSI and
Increases Neuronal Viability in Vivo. We wished to determine whether PSI and neuronal death following in vivo ischemia were
associated with calpain activation and loss of eIF4G1; to this end
we used the rat four-vessel occlusion model of global ischemia.
In this model, a brief period of global ischemia induces delayed
neurodegeneration preferentially in CA1 pyramidal neurons of
the hippocampus (22). Twenty-four hours after ischemia induced
by 12-min four-vessel occlusion (23), α-spectrin in CA1 was
largely cleaved to the 145-kDa fragment. This effect was pre-
vented by the calpain inhibitor D6 8170 (see SI Materials and
Methods for details on drug administration), which also increased
full-length α-spectrin 24 h after ischemia (Fig. S9 A and B). 

Protein synthesis determined by autoradiography and quantifi-
cation of 14Cleucine uptake was markedly depressed in CA1 at
24 h and 8 wk after 12 min ischemia (Fig. 5A). In contrast, there
was no decrease in protein synthesis in hippocampal CA3 or
cortex at these times. Calpain inhibition prevented the de-
pression of protein synthesis at both 24 h and 8 wk (Fig. 5A, B,
and D and Table S1). Importantly, neuronal viability was also
increased in the CA1 field of rats treated with the calpain
inhibitor at 24 h and 8 wk (Fig. 5 C and E), as was recently shown
with shRNA-mediated knockdown of μ-calpain (24). Levels of
eIF4G1 in CA1 were also reduced in the vulnerable CA1 but not
in the resistant CA3; the reduction in CA1 was largely prevented
by the calpain inhibitor D6 8170 (Fig. 5F). In total, these results recapitulate our in vitro findings demonstrating that
increased calpain activity following ischemia in vivo is responsible
for decreased levels of eIF4G1, persistent PSI, and ultimately
delayed neuronal death.

Discussion

It has been known for nearly 40 y that protein synthesis is
inhibited in the entire forebrain following global ischemia (7)
and that persistent inhibition of protein synthesis is a robust
predictor of eventual neuronal death in both global (8) and focal
(9) ischemia. Despite this knowledge, protein synthesis inhibition
has yet to be directly linked to neuronal death. In fact, persistent PSI was thought to be an epiphenomenon of ischemic injury that persisted due to repeated peri-infarct depolarizations or to be secondary to energy failure (9).

Here we provide compelling evidence of a direct link between PSI and ischemic neuronal death. The proposed mechanistic connection between ischemia and PSI is the pathological degradation of eIF4G1 mediated by ischemia-induced calpain activation. Calpain inhibition largely prevented ischemia-induced decreases in eIF4G1, allowing for recovery of protein synthesis. Maintenance of eIF4G1 levels by overexpression or calpain inhibition resulted in increased neuronal viability that was associated with increased cap-dependent protein synthesis.

To our knowledge, this is a unique finding demonstrating that degradation of a translation initiation factor is directly involved in neuronal death. The decrease in overall translation rate caused by multiple death stimuli is known to correlate with eIF4G1 levels (25). Decreases in eIF4G1 have also been implicated in neuronal death in previous in vivo studies that demonstrated decreased levels of eIF4G1 for up to 4 h of reperfusion in global ischemia models (5) and for up to 24 h following focal ischemia (18). Importantly, the loss of eIF4G1 at 24 h after focal ischemia (18) is highly correlated with the eventual loss of brain tissue after ischemia wherein loss of PSI (likely due to transient P-eIF2α up-regulation), they eventually restored translation by preserving levels of eIF4G1. This result is an important distinction in terms of neuroprotective strategies, as the aim for clinical neuroprotection is to prevent the eventual loss of brain tissue after ischemia wherein loss of eIF4G1 and initial PSI has already occurred.

Restoration of protein synthesis is clearly important for maintenance of neuronal viability following ischemia. Neuroprotection provided by eIF4G1 overexpression in vitro and MDL 28170 in vivo significantly increased protein synthesis; however, neuroprotection by eIF4G1 may be only partially due to restoration of translation, as treatment with CHX did not completely
block the neuroprotective effect. Other mechanisms may therefore be involved in the protective effect of elf4G1. It was recently demonstrated that elf4G1 was involved with preservation of bioenergetics and mitochondrial activity along with suppression of autophagy (21). The potential role of elf4G1 in regulating mitochondrial biogenesis and autophagy via protein synthesis-dependent and -independent mechanisms is an enticing area for further study.

In conclusion, we provide evidence that levels of a translation initiation factor are directly linked to cell viability. We show that calpain-mediated degradation of elf4G1 is a causal link between ischemia and persistent PSI leading to neuronal death. Preservation of elf4G1 levels promotes recovery from ischemia-induced PSI and provides neuroprotection. Additional assessments of the types of mRNA translated and the mechanism of translation following ischemia are also warranted. Further understanding of how elf4G1 regulates persistent PSI and contributes to the multitude of factors causing postischemic neurodegeneration may lead to the development of new neuroprotective strategies.

### Materials and Methods

Details beyond the descriptions here are provided in SI Materials and Methods.

#### Neuronal Ischemia Models.

All animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rat primary cortical neuronal cultures were derived from E18 embryos of Sprague-Dawley rats, and in vitro ischemia was induced in neurons by oxygen and glucose deprivation. Global ischemia was induced for 12 min in anesthetized male Sprague-Dawley rats under isoflurane anesthesia using the four-vessel occlusion model (23). Cell death and protein synthesis of hippocampal CA1 neurons were quantified at 24 h, 4 d, or 8 wk after ischemia (23).

#### Cell Viability Assays.

Neuronal viability was assessed with Alamar Blue, Hoechst 33342 staining and lactate dehydrogenase (LDH) release as previously described (23) with slight modifications (SI Materials and Methods). The amount of LDH release was presented as percentage of control.

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