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Phosphorylation of the Translation Initiation Factor eIF2α Increases BACE1 Levels and Promotes Amyloidogenesis

Tracy O'Connor[1], **Katherine R. Doherty-Sadleir**[1], **Erika Maus**[1], **Rodney A. Velliquette**[1], **Jie Zhao**[1], **Sarah L. Cole**[1], **William A. Eimer**[1], **Brian Hitt**[1], **Leslie A. Bembinster**[1], **Sven Lammich**[2], **Stefan F. Lichtenthaler**[2], **Sébastien S. Hébert**[3], **Bart De Strooper**[3], **Christian Haass**[2], **David A. Bennett**[4], and **Robert Vassar**[1,†]

1*Department of Cell & Molecular Biology, Northwestern University Feinberg School of Medicine, Chicago, IL*

2*Center for Integrated Protein Science Munich and Adolf-Butenandt-Institute, Department of Biochemistry, Laboratory for Neurodegenerative Disease Research, Ludwig-Maximilians-University, Munich, Germany*

3*Center for Human Genetics, Katholieke Universiteit Leuven and Department of Molecular and Developmental Genetics, VIB, Leuven, Belgium*

4*Rush Alzheimer's Disease Center, Rush University Medical Center, Chicago, IL*

Summary

Beta-site APP cleaving enzyme-1 (BACE1), the rate-limiting enzyme for β-amyloid (Aβ) production, is elevated in Alzheimer's disease (AD). Here, we show that energy deprivation induces phosphorylation of the translation initiation factor eIF2α eIF2α-P), which increases the translation of BACE1. Salubrinal, an inhibitor of eIF2α-P phosphatase PP1c, directly increases BACE1 and elevates Aβ production in primary neurons. Preventing eIF2α phosphorylation by transfection with constitutively active PP1c regulatory subunit, dominant negative eIF2α kinase PERK, or PERK inhibitor $P58^{IPK}$ blocks the energy deprivation-induced BACE1 increase. Furthermore, chronic treatment of aged Tg2576 mice with energy inhibitors increases levels of eIF2α-P, BACE1, Aβ, and amyloid plaques. Importantly, eIF2α-P and BACE1 are elevated in aggressive plaque-forming 5XFAD transgenic mice, and BACE1, eIF2α-P, and amyloid load are all correlated in humans with AD. These results strongly suggest that eIF2α phosphorylation increases BACE1 levels and causes Aβ overproduction, which could be an early, initiating molecular mechanism in sporadic AD.

Introduction

AD is a devastating neurodegenerative brain disorder of the elderly for which no cure or disease-modifying treatment exists. A major histopathological hallmark of AD is the amyloid plaque, composed of the Aβ peptide (Glenner and Wong, 1984). Aβ appears to play a critical, early role in the pathogenesis of AD, because all known familial forms of AD (FAD) result from autosomal dominant mutations that enhance pro-amyloidogenic processing of APP (Hardy and Selkoe, 2002; Younkin, 1998). Although the mechanisms of Aβ overproduction in FAD are fairly well understood, little is known about the cause(s) of sporadic AD (SAD),

†Corresponding Author: Robert Vassar, PhD, Department of Cell & Molecular Biology, Northwestern University Feinberg School of Medicine, 303 E. Chicago Ave., Chicago, IL 60611, Phone: (312) 503-3361, E-mail: r-vassar@northwestern.edu.

which is the predominant form of AD. However, based on the strong genetic association of Aβ with FAD and the clinical and pathological similarities between FAD and SAD, it is likely that Aβ is also involved in SAD pathogenesis at an early stage.

Aβ is generated from the sequential proteolytic processing of amyloid precursor protein (APP) by the enzymes β- and γ-secretase (De Strooper, 2003; Sisodia and St George-Hyslop, 2002). The β-secretase was identified as the transmembrane aspartic protease, β-site APP cleaving enzyme 1 (BACE1), responsible for initiating cleavage of APP to form Aβ (Hussain et al., 1999; Lin et al., 2000; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999). Because of its central role in the amyloidogenic process, BACE1 is a promising drug target for AD therapy (Laird et al., 2005; Luo et al., 2001; Ohno et al., 2006; Ohno et al., 2004; Singer et al., 2005; Vassar et al., 1999). The K670N, M671L (Swedish) mutation in APP (APP_{sw}) promotes cleavage of APP by BACE1, increases total Aβ production, and causes FAD (Citron et al., 1992), together implying that increased BACE1 activity might be sufficient to induce AD pathogenesis. Importantly, recent studies have shown that BACE1 levels and activity are increased in post-mortem AD brain samples (Fukumoto et al., 2002; Holsinger et al., 2002; Li et al., 2004; Sennvik et al., 2004; Tyler et al., 2002; Yang et al., 2003, Zhao et al., 2007), suggesting that elevated BACE1 levels in the brain may play a role in the development of SAD. Most studies show that BACE1 mRNA levels are not increased in AD brain, indicating a post-transcriptional mechanism causes the BACE1 increase.

One factor that appears closely associated with AD is impaired cerebral energy metabolism. Positron emission tomography (PET) imaging studies have shown that glucose utilization is dramatically lower in AD brain than in age-matched, non-demented brain (reviewed in (de Leon et al., 2007; Mosconi et al., 2007). Moreover, post-mortem analysis of AD brain shows down-regulated expression of mitochondrial enzymes (de Leon et al., 1983; Rapoport, 1999a, b), indicating that energy metabolism may be deficient in AD. Aging, the primary risk factor for SAD, is also a major risk factor for cardio- and cerebrovascular disease (Cole and Vassar, 2008; Decarli, 2004), implying that low-level chronically reduced glucose and oxygen delivery to the brain may contribute to the development of AD. Importantly, young and middle-aged non-demented carriers of the apolipoprotein E4 (ApoE4) allele, a major genetic risk factor for AD, and patients with mild cognitive impairment (MCI), a condition that precedes clinical AD, also exhibit reduced brain glucose utilization by PET imaging (Reiman et al., 2004; Wolf et al., 2003), suggesting that impaired cerebral energy metabolism may be an early event in AD pathogenesis rather than a downstream consequence of neurodegeneration.

We have shown previously, using an acute pharmacological model of energy metabolism inhibition in pre-plaque APP-overexpressing transgenic mice (Tg2576), that BACE1 and Aβ levels become elevated post-transcriptionally in the brain, indicating that energy deprivation may be amyloidogenic *in vivo* (Velliquette et al., 2005). These data lend further support to a growing body of evidence that BACE1 may play a normal physiological role in the cellular stress response (Kamenetz et al., 2003; Tamagno et al., 2005; Tesco et al., 2007; Velliquette et al., 2005; Wen et al., 2004; Wen et al., 2008; Yang et al., 2003; Zhao et al., 2007). Two different hypotheses have emerged to explain how BACE1 might be post-transcriptionally regulated in response to stress or aging: alterations in microRNA profiles and apoptosis-dependent changes in BACE1 protein stability (Hebert et al., 2008; Tesco et al., 2007; Wang et al., 2008). One other area that remains unexplored is the possible role of stress-induced alterations in the translational control of the BACE1 transcript. Stress-induced changes in the translation initiation machinery and a subsequent shift to selective translation of stress-response transcripts is a widely known phenomenon (Holcik and Sonenberg, 2005; Pavitt, 2005; Schroder and Kaufman, 2006). Furthermore, recent studies have shown that the BACE1 mRNA 5'-untranslated region (5'UTR) acts as a translational repressor (De Pietri Tonelli et al., 2004; Lammich et al., 2004; Mihailovich et al., 2007; Rogers et al., 2004). This property of

the BACE1 mRNA 5' UTR appears to be related to the fact that it is long, GC-rich, possesses extensive secondary structure, and contains 3 upstream open reading frames (uORFs), characteristics shared with many 5' UTRs of transcripts that are translationally controlled by cellular stress. In addition, it has been shown that regulation of energy stores and translational control of proteins are intricately related (Harding et al., 2001; Lee, 2001; Scheuner et al., 2001). Taken together, these results suggest the intriguing possibility that the BACE1 increase in AD may be the result of translational de-repression of the BACE1 mRNA 5' UTR induced by energy metabolism stress, a mechanism that could have important implications for AD pathogenesis.

Here, in order to identify the molecular mechanism underlying elevated BACE1 levels in response to energy inhibition, we used glucose deprivation in cell culture as an *in vitro* model of energy deficiency. In stable BACE1-expressing HEK-293 cells and primary mouse cortical neurons, glucose deprivation caused a post-transcriptional increase in BACE1 level that correlated with enhanced phosphorylation of the translation initiation factor eIF2α, a well-established mechanism of stress-induced translational control (Clemens, 2001; Schroder and Kaufman, 2006). Direct pharmacologic induction of eIF2α phosphorylation elevated BACE1 levels and enhanced Aβ production, and conversely, genetic inhibition of eIF2α phosphorylation prevented the BACE1 increase under conditions of energy deprivation *in vitro*. Additionally, chronic energy deprivation *in vivo* increased eIF2α-P and BACE1 levels, and exacerbated amyloid pathology in Tg2576 mice. Elevated eIF2α-P and BACE1 levels were also observed in another APP transgenic with aggressive amyloid pathology (5XFAD) and in human AD brain. We conclude that eIF2α phosphorylation increases BACE1 levels via a translational control mechanism and promotes amyloidogenesis in response to energy deprivation, and that this mechanism may play an important role in sporadic AD pathogenesis.

Results

Energy Deprivation Increases BACE1 Levels by a Translational Mechanism

Previously, we observed increased BACE1 protein levels in the absence of increased BACE1 mRNA levels following acute energy inhibitor treatment in mice, suggesting a post-transcriptional mechanism (Velliquette et al., 2005). To investigate the molecular mechanism responsible for raising the BACE1 level in response to energy inhibition, we used HEK-293 cells that stably overexpress the entire 2.5kb human BACE1 transcript, including the complete 5' and 3' UTRs, driven by the CMV promoter (BACE1-293 cells). BACE1-293 cells are ideal for investigating BACE1 post-transcriptional mechanisms because the cell line expresses BACE1 mRNA from a constitutive heterologous promoter that is transcriptionally stable under a wide range of conditions, and it expresses BACE1 mRNA with the endogenous 5' and 3' UTRs that are required for the normal translational regulation of BACE1 mRNA. We treated BACE1-293 cells for 2 to 48 hrs in media containing 25mM glucose (CON) or lacking glucose (NG) and analyzed BACE1 levels in lysates by immunoblot (Fig. 1A, B). Energy deprivation slowed cell growth but was otherwise non-toxic, as indicated by failure to activate caspase 3 (Fig. S2). BACE1 levels in NG-treated cells showed a clear upward trend at 2 hrs that became statistically significant at 6 hrs and peaked around 12 hrs (Fig. 1A, B; $p < 0.001$). The glucose deprivation-induced BACE1 increase plateaued at ~150-170% of control (CON) levels and remained high for the duration of the experiment. Importantly, the early increase in BACE1 levels following glucose deprivation suggested a post-transcriptional mechanism.

Although the constitutively active CMV promoter made it unlikely that increased BACE1 gene transcription was the cause of the glucose deprivation-induced BACE1 elevation, we sought to definitively exclude this possibility by treating BACE1-293 cells with the transcriptional inhibitor actinomycin D (ActD) in the presence or absence of glucose (Fig. 1C-E). First, it is relevant to note that 12 hrs of NG treatment of BACE1-293 cells on its own did not increase

steady-state BACE1 mRNA levels relative to control treated cells (Fig. 1E, two left bars), even though BACE1 protein levels were significantly elevated (Fig. 1D, two left bars). Most importantly, ActD treatment failed to prevent BACE1 levels from increasing in cells incubated in NG medium. BACE1 was increased to the same level in NG-treated cells regardless of whether they were exposed to ActD or not (Fig. 1C-D). This is a striking result, given that ActD dramatically reduced BACE1 mRNA levels in cells either incubated with or without glucose, as determined by BACE1 primer-specific TaqMan quantitative real-time PCR (Fig. 1E, two right bars). These results conclusively demonstrate that the BACE1 increase induced by energy deprivation in BACE1-293 cells was the result of a post-transcriptional mechanism and was not due to either elevated BACE1 transgene mRNA synthesis or increased BACE1 mRNA stability.

Recent studies have shown that BACE1 protein stability can be modulated by the lysosomal and ubiquitin-proteasomal degradation pathways (Koh et al., 2005; Qing et al., 2004; Tesco et al., 2007). To determine whether increased BACE1 protein stability was the post-transcriptional mechanism underlying the BACE1 increase in response to energy deprivation, we used pulse-chase ^{35}S-metabolic radiolabeling to measure the half-life ($t_{1/2}$) of BACE1 protein in BACE1-293 cells incubated under normal chase conditions compared to cells chased in NG media or in media containing 2-deoxyglucose (2DG), which competes with glucose for catabolism by hexokinase. We observed that the normal $t_{1/2}$ of BACE1 is ~12 hours, similar to previous reports (Haniu et al., 2000; Huse et al., 2000). Importantly, under NG or 2DG chase conditions, we found that the $t_{1/2}$ of BACE1 was similar to control (Fig. 1F), indicating that increased BACE1 protein stability is not the post-transcriptional mechanism responsible for elevated BACE1 levels in response to energy deficiency.

Taken together, our results thus far excluded gene transcription, mRNA stability, and protein stability as causes of the energy deprivation-induced BACE1 increase, suggesting that the BACE1 increase was likely to occur by a translational mechanism. In support of this notion, recent studies have shown that translation is inhibited by the BACE1 mRNA 5' UTR, which is long (453 nts), GC-rich (77%), is predicted to have extensive secondary structure, and contains 3 uORFs (De Pietri Tonelli et al., 2004; Lammich et al., 2004; Mihailovich et al., 2007; Rogers et al., 2004). These characteristics of the BACE1 5'UTR are typical of translationally-regulated stress response mRNAs (Clemens, 2001), suggesting that the BACE1 transcript may be a target of translational control by one or more stress-activated pathways. However, previous studies had not addressed whether BACE1 was regulated by stress-induced translational pathways, and if so, whether BACE1 translational control could be relevant to AD pathogenesis. To initially test these hypotheses, we investigated whether the BACE1 mRNA 5'UTR was required for the energy deprivation-dependent BACE1 increase. We transiently transfected HEK-293 cells with CMV promoter-driven expression vectors encoding BACE1 either with (+5'UTR) or without (-5'UTR) the BACE1 5'UTR (Lammich et al., 2004) and then treated cultures in normal or NG media for 24 hrs (Fig. 1G, H). Similar to our BACE1-293 cell experiments, transfection with the BACE1 +5'UTR construct followed by BACE1 immunoblot analysis revealed a ~150% increase of BACE1 levels when cells were incubated in NG media, compared to control glucose-containing media (Fig. 1G, H, left). Removal of the BACE1 5'UTR caused an increase in BACE1 levels in transfected cells (Fig. 1G, H, right), as previously reported (Lammich et al., 2004). In addition to the mature ~70kDa BACE1 species, a significant proportion of the BACE1 in cells transfected with the BACE1 -5'UTR construct migrated at ~60kDa on immunoblots. Since the ~60kDa BACE1 species also labeled with an anti-BACE1 C-terminal antibody (not shown), it was full-length BACE1 rather than a truncated BACE1 fragment and therefore was likely to be immaturely glycosylated BACE1 (Haniu et al., 2000). Apparently, the increased expression of BACE1 from the BACE1 -5'UTR construct overloaded the ER, causing accumulation of immature BACE1. Importantly, transfection with the BACE1 -5'UTR construct followed by glucose

deprivation failed to cause BACE1 levels to increase above those observed in BACE1 -5'UTR transfected cells incubated in glucose-containing media (Fig. 1G, H, right). As expected, glucose deprivation did not affect levels of BACE1 mRNA transcribed from BACE1 +5'UTR and -5'UTR expression vectors, as measured by TaqMan RT-PCR analysis (not shown). Although unlikely, at this point we cannot exclude the possibility that the glucose deprivation-induced BACE1 increase was masked by the large rise in BACE1 expression from the BACE1 -5'UTR construct. However, our results strongly suggest that the BACE1 mRNA 5' UTR is necessary for the energy deprivation-dependent increase of BACE1 and further indicate that a translational control mechanism is at play.

Energy Deprivation Increases eIF2α Phosphorylation

Control of translation initiation is considered to be of prime importance for translational regulation. It is well established that diverse cellular stresses affect translation at the level of initiation (Clemens, 2001). Since our experiments demonstrated that the BACE1 mRNA 5' UTR was required for the energy deprivation-induced BACE1 increase, and that the BACE1 5'UTR exhibited characteristics of translationally-regulated stress response mRNAs, we investigated whether BACE1 levels might be regulated by stress-induced translational control pathways during energy deprivation. Initially, we screened cell lysates from NG-treated and control BACE1-293 cells by immunoblot for phosphorylation of proteins involved in translational regulation following stress (Figs. 2, S1). Using this screen we found that phosphorylation of serine 51 of the α subunit of eukaryotic translation initiation factor 2 (eIF2α-P(Ser51)) was dramatically increased in response to NG treatment, implicating this component of the translation initiation complex in the energy deprivation-induced BACE1 elevation. Strikingly, 24 hrs of NG treatment increased the ratio of eIF2α-P(Ser51) to total eIF2α (eIF2α-T) to ~250% of cells grown in normal glucose-containing media (Fig. 2A,B; $p < 0.01$). Notably, eIF2α-P levels were increased to ~200% of control at only 2 hrs of NG treatment, a time when BACE1 levels had just begun to rise but were not yet significantly elevated. Therefore, NG-induced eIF2α phosphorylation preceded the BACE1 increase, as expected for a cause-and-effect relationship.

We found no evidence of alterations in the phosphorylation state of the other major factor that is involved in the control of translation initiation during stress, phosphorylated eukaryotic translation initiation factor 4E (eIF4E-P; Fig 2A, B), indicating that energy deprivation specifically increases phosphorylation of eIF2α. In addition, treatment of BACE1-293 cells with rapamycin, an inhibitor of the serine/threonine kinase mammalian target of rapamycin (mTOR), had no effect on BACE1 levels (Fig. S1). mTOR is the kinase that regulates eIF4E binding protein (4E-BP), eukaryotic elongation factor 2 (eEF2) kinase, and the ribosomal subunit protein S6 kinase. S6 and eEF2 are in turn phosphorylated and regulated by S6 kinase and eEF2 kinase, respectively. Therefore, the negative rapamycin results further exclude a role for eIF4E and indicate lack of involvement of eEF2 and S6 in translational control of BACE1.

We also found no evidence of caspase-3 activation in glucose-deprived BACE1-293 cells, indicating that apoptosis is not involved in the BACE1 increase (Fig. S2). We did observe increased phosphorylation of the stress-activated proteins c-Jun and c-Jun N-terminal kinase (JNK) in response to glucose deprivation (Fig 2A,B), which may simultaneously lead to transcriptional activation of other stress response proteins. Interestingly, we also observed increased levels of total c-Jun protein, the structural and functional homolog of the yeast protein GCN4 (Struhl, 1987;Vogt et al., 1987) which is a well-established translational target of the eIF2α-P pathway (Fig 2A,B; (Cigan et al., 1993;Vazquez de Aldana et al., 1993). Although it is unknown whether eIF2α-P regulates translation of c-Jun mRNA, several studies report that c-Jun mRNA translation is increased under different conditions of stress or injury (Gietzen et al., 2004;Polak et al., 2006;Rao, 2000;Spruill et al., 2008;Vardimon et al., 2006). Taken

together, these results demonstrate that eIF2α phosphorylation is specifically increased as a result of energy deprivation and is correlated with the BACE1 increase.

Selective Inhibition of eIF2α Dephosphorylation Increases BACE1 Levels

Phosphorylation of eIF2α on serine 51 is a major mechanism that regulates initiation of translation in response to various cellular stresses, including virus infection, nutrient deprivation, iron deficiency, and accumulation of unfolded proteins in the ER (Clemens, 2001; Proud, 2001). Depending on the specific cellular stress, eIF2α is phosphorylated by at least 4 different kinases, including double-stranded RNA-activated kinase (PKR), general control nonderepressible 2 kinase (GCN2), heme-regulated inhibitor kinase (HRI), and PKR-like ER kinase (PERK). Following stress-induced phosphorylation of eIF2α, translation of normal cellular mRNAs is repressed, while the translational initiation of select mRNAs involved in stress response is stimulated. eIF2α-P is dephosphorylated by protein phosphatase-1 (PP1) complexed with its regulatory subunit, growth arrest and DNA damage-inducible protein 34 (GADD34). Importantly, the PP1/GADD34 complex is inhibited by the small molecule drug salubrinal (Sal), which selectively blocks dephosphorylation of eIF2α-P but not other PP1 substrates (Boyce et al., 2005).

If eIF2α phosphorylation stimulates the translation of BACE1 mRNA, then directly raising levels of eIF2α-P by selective inhibition of eIF2α-P dephosphorylation with Sal should increase BACE1 levels in the absence of energy deprivation. To test this hypothesis, we treated BACE1-293 cells for 24 hrs with Sal (100μM), NG medium, or glucose-containing medium. Like NG treatment, incubating BACE1-293 cells with salubrinal caused both eIF2α-P(Ser51) and BACE1 levels to increase to ~150-200% of control values (Fig. 3A,B; $p < 0.05$). Therefore, PP1/GADD34 inhibition raised eIF2α-P levels and directly caused the BACE1 increase without the need of glucose deprivation, demonstrating that BACE1 is a translational target of the stress-activated eIF2α-P(Ser51) pathway.

Blocking eIF2α Phosphorylation by Constitutive Activation of the PP1/GADD43 Complex Prevents the Energy Deprivation-Induced BACE1 Increase

To further establish the role of eIF2α in BACE1 translational control, we sought to determine the effects of directly blocking eIF2α phosphorylation on the energy deprivation-induced BACE1 increase. For this we used a construct encoding only the C-terminal activation domain of GADD34 that lacks the N-terminal regulatory domain (GADD34ΔN) and thus constitutively activates PP1 (Novoa et al., 2001). Therefore, upon transfection GADD34ΔN should prevent the increases of both eIF2α-P and BACE1 following glucose deprivation. To test this, we transiently transfected BACE1-293 cells with expression constructs encoding either GADD34ΔN or an inactive GADD34 construct lacking the C-terminal PP1 activation domain, which has no effect on PP1 activity and served as a transfection control. Following overnight recovery, GADD34ΔN and control-transfected cells were treated for 12 hrs in NG or glucose-containing media, and cell lysates were analyzed by immunoblot for BACE1 and eIF2α-P. Control-transfected BACE1-293 cells exhibited the expected increase in levels of BACE1 and eIF2α-P(Ser51) levels in response to NG treatment (Fig. 3C, middle four lanes; 3D). In contrast, GADD34ΔN-transfected cells incubated in NG media showed no increase in eIF2α-P(Ser51) or BACE1 levels in response to NG treatment (Fig. 3C, right four lanes; 3D) compared to control transfection, demonstrating that inhibition of eIF2α phosphorylation completely blocks NG-induced BACE1 elevation. Together with our salubrinal experiments, these results clearly demonstrate that the rise in BACE1 level in response to energy deprivation is the result of increased phosphorylation of eIF2α at Ser51.

Activation of the eIF2α Kinase PERK is Necessary for the Energy-Deprivation Induced BACE1 Increase

Of the four known eIF2α kinases, the two most likely to be induced by energy metabolism stress are PERK and GCN2, which are activated by the ER stress/unfolded protein response (UPR) and amino acid deprivation, respectively. PKR and HRI are less likely to play a role in the energy deprivation-induced BACE1 increase because they are activated by virus infection and heme deficiency in erythrocytes, respectively (Clemens, 2001). Therefore, we sought to determine whether PERK or GCN2 was responsible for phosphorylating eIF2α and causing the BACE1 increase following glucose deprivation. To accomplish this, we used kinase-dead forms of PERK and GCN2 that had a C-terminal deletion (PERK; Harding et al., 1999) or were mutated at the critical lysine 618 residue in the conserved kinase domain of the enzyme (GCN2; Sood et al., 2000), thus producing dominant-negative molecules that block eIF2α phosphorylation. We transiently transfected BACE1-293 cells with constructs encoding dominant negative PERK (PERKDN) or GCN2 (GCN2DN), allowed cells to recover overnight, and then treated cells for 12 hrs in NG or glucose-containing media. Expression of GCN2DN in combination with glucose deprivation failed to prevent eIF2α phosphorylation or BACE1 elevation in BACE1-293 cells (Fig 3G, H), indicating that GCN2 is not the kinase that phosphorylates eIF2α under glucose-deficient conditions. In contrast, expression of PERKDN in combination with NG treatment completely blocked the increases of both eIF2α-P and BACE1 (Fig 3E, F), demonstrating that PERK is the specific kinase responsible for phosphorylating eIF2α and controlling the energy deprivation-induced BACE1 elevation.

To provide further support for the role of PERK in BACE1 translational control, we performed two additional experiments using a cellular inhibitor of PERK, $P58^{IPK}$ (Yan et al., 2002), and the compound tauroursodeoxycholic acid (TUDCA), which alleviates ER stress (Ozcan et al., 2006). The unfolded protein/ER stress response induces the expression of $P58^{IPK}$, a molecular chaperone that is thought to bind to the kinase domain of PERK and inhibit its activity (Yan et al., 2002). Transient transfection of BACE1-293 cells with an expression vector encoding $P58^{IPK}$ prevented the BACE1 increase following glucose deprivation (Fig. S3A, B), mirroring our previous results with PERKDN transfection. Similarly, TUDCA treatment of BACE1-293 cells also blocked the BACE1 increase caused by incubation in NG medium (Fig. S3C, D). Taken together, our experiments with PERKDN, GCN2DN, $P58^{IPK}$, and TUDCA strongly suggest that PERK is the eIF2α kinase activated by energy deprivation, and that it is ultimately responsible for the eIF2α-P induced translational increase of BACE1.

Glucose Deprivation Causes eIF2α Phosphorylation and Increases BACE1 Levels in Cultured Primary Neurons by a Post-Transcriptional Mechanism

Thus far in our analysis, we had demonstrated that PERK and phosphorylation of eIF2α were responsible for the energy deprivation-induced BACE1 increase in BACE1 overexpressing HEK-293 cells. We next wanted to show that the same translational control mechanism was involved in the regulation of endogenous BACE1 in neurons. To accomplish this, we cultured C57/BL6 mouse primary cortical neurons for 7 days *in vitro* (DIV) and then treated cultures for up to 48 hrs in NG or glucose-containing media. Neuron lysates were prepared and analyzed for endogenous BACE1 levels by immunoblot. BACE1 levels were increased in primary neuron cultures by 24 hrs of treatment in NG media (not shown) and peaked at ~36 hrs (Fig. 4A, B; ~150% of control, $p < 0.05$). Unexpectedly, TaqMan quantitative PCR analysis of mRNA isolated from parallel primary neuron cultures showed a *decrease* in BACE1 transcript levels in response to NG media (Fig. 4C; $p < 0.05$), clearly demonstrating that a post-transcriptional mechanism was responsible for the BACE1 increase following NG treatment. Similar to BACE1-293 cells, the eIF2α-P(Ser51):eIF2α total ratio in 36 hr NG-treated primary neurons was elevated to ~150% of control values (Fig. 4A, B; $p < 0.05$) indicating that

eIF2α phosphorylation was the likely mechanism of the BACE1 protein elevation in neurons as well.

Selective Inhibition of eIF2α Dephosphorylation Increases BACE1 Level and Aβ production in Tg2576 Primary Neurons

If eIF2α phosphorylation increases the translation of BACE1 mRNA in neurons as it does in BACE1-293 cells, then directly raising levels of neuronal eIF2α-P by selective inhibition of eIF2α-P dephosphorylation with salubrinal treatment should elevate BACE1 levels in the absence of energy deprivation. Moreover, the resulting rise in BACE1 level should cause an increase in neuronal Aβ generation as well. To test these hypotheses, we cultured primary cortical neurons from Tg2576 transgenic mice that overexpress human APPsw (Hsiao et al., 1996) and treated the cultures with salubrinal. Since the effects of salubrinal on primary neurons had not yet been published, we chose two salubrinal concentrations (50μM and 80μM) that we determined from a previous study (Boyce et al., 2005) would be likely to inhibit PP1/GADD34 with minimal neuronal toxicity. Tg2576 primary neurons from E15.5 embryos were cultured for 7 DIV, treated with 50μM or 80μM salubrinal for 48 hrs, and conditioned media and neuronal lysates collected. As we observed in BACE1-293 cells, immunoblot analysis revealed that salubrinal treatment increased levels of both eIF2α-P(Ser51) and endogenous BACE1 protein in Tg2576 neurons (Fig. 4D, E). Furthermore, these results together with the glucose deprivation experiments establish that the same mechanism of BACE1 increase occurs in both BACE1-293 cells and primary neurons.

Next, we determined whether salubrinal treatment of the Tg2576 neuron cultures had increased Aβ production. To do so, we used a human $A\beta_{40}$-specific ELISA to measure levels of the 40-amino acid isoform of Aβ ($A\beta_{40}$) secreted into the conditioned media of control, 50μM, and 80μM Sal-treated neuron cultures. Changes in BACE1 activity affect the generation of all isoforms of Aβ equally (Vassar et al., 1999). $A\beta_{40}$ is the most abundant Aβ isoform produced in cells and tissues, and it is a gauge of BACE1 activity in cells. In addition, $A\beta_{40}$ is the major isoform of Aβ produced in Tg2576 transgenic mice. As expected, $A\beta_{40}$ levels were significantly elevated in conditioned media from salubrinal-treated neurons, as compared to control (Fig 4F; CON = 13.25 ± 0.33 ng $A\beta_{40}$/mg protein; 50μM Sal = 15.88 ± 0.63, $p < 0.05$; 80μM Sal = 27.19 ± 1.61, $p < 0.01$).

The $A\beta_{40}$ increase for the 50μM Sal-treated Tg2576 neurons was less than expected, based on the level of phosphorylated eIF2α (Fig. 4E, F). The reason for this is unclear, but we suspect that BACE1 levels increased at a lower rate in 50μM Sal compared to 80μM Sal, thereby delaying the $A\beta_{40}$ increase. It is likely that $A\beta_{40}$ levels in 50μM Sal treated cultures would have eventually caught up with those of 80μM Sal. Regardless, it is important to note that the $A\beta_{40}$ increase in 50μM Sal-treated Tg2576 neurons was significant, and that the $A\beta_{40}$ increase for 80μM Sal was ~2-fold over control. In addition, our results demonstrate that selective inhibition of eIF2α dephosphorylation directly causes BACE1 levels to increase in neurons in the absence of energy deprivation. Taken together, the salubrinal-treated Tg2576 neuron cultures confirm that BACE1 is a translational target of eIF2α-P(Ser51) in neurons and demonstrate that direct induction of eIF2α-P increases Aβ production, which has important implications for AD pathogenesis.

Chronic Energy Inhibition Increases eIF2α Phosphorylation, BACE1 Levels, and Amyloidogenesis in Tg2576 Mice

Thus far, our results demonstrated that energy deprivation caused increases in eIF2α phosphorylation, BACE1 translation, and Aβ production *in vitro*, conditions that we predicted would exacerbate amyloid deposition *in vivo*. To test this, we designed a chronic *in vivo* energy deprivation paradigm based on our previous acute study in which we treated Tg2576 mice with

inhibitors of energy metabolism that caused increases in BACE1 level and Aβ production (Velliquette et al., 2005). In that acute study, we gave 2 month-old Tg2576 mice single intraperitoneal (i.p.) injections of compounds that produced or mimicked hypoglycemia, inhibited ATP synthesis, or caused neuronal over-excitation which depleted ATP stores: insulin, 2-deoxyglucose (2DG) and 3-nitropropionic acid (3NP), and kainic acid (KA). Since our *in vitro* studies primarily focused on the effects of glucose deprivation on eIF2α phosphorylation and BACE1 level elevation, in the chronic *in vivo* study we chose to only use the two compounds that would specifically interfere with glucose energy metabolism at the level of glycolysis and the Krebs cycle: 2DG and 3NP. 2DG is a glucose analog that is a competitive inhibitor of hexokinase, while 3NP inhibits the Krebs cycle and electron transport chain enzyme succinate dehydrogenase. Insulin and KA were not used in the current study because we reasoned that these two compounds were more likely to cause pleotrophic physiological effects *in vivo*, especially under conditions of chronic dosing, thus potentially complicating the interpretation of results.

For our chronic *in vivo* energy deprivation experiment, 9 month-old Tg2576 and C57/BL6 (wild-type) mice were treated once per week with i.p. injections of 2DG (1g/kg), 3NP (80mg/kg), or vehicle for a duration of 3 months. In our acute study, BACE1 and Aβ levels stayed elevated for at least a week following a single dose of 2DG or 3NP (Velliquette et al., 2005), thus providing the rationale for single weekly injections in the current study. Treatments were started at 9 months of age, when Tg2576 mice were on the cusp of amyloid plaque formation (Hsiao et al., 1996), and concluded when mice were 12 months old. The doses of 2DG and 3NP were chosen based on a pilot study showing that they were able to increase BACE1 to levels similar to those observed in our acute study (not shown). These doses were well-tolerated by the mice, no mortality was observed, and treatments caused only temporary lethargy lasting 30-60 min. Cresyl violet staining and GFAP immunohistochemistry of brain sections from 2DG or 3NP treated mice revealed no significant neurodegeneration or gliosis associated with treatment (Fig. S4).

Following 3 months of treatment, hemibrains of each Tg2576 mouse were analyzed for levels of eIF2α-P, BACE1 (by immunoblot) and $A\beta_{40}$ (ELISA), and amyloid plaque number (by anti-Aβ immunohistochemistry and Thioflavin-S staining). C57/BL6 hemibrains were analyzed in a similar manner as those of Tg2576, except one hemibrain from each C57/BL6 mouse was processed for TaqMan mRNA quantification rather than histology. As expected, chronic experimental glucose deprivation caused a significant increase of BACE1 level in the brains of Tg2576 (Fig. 5A, C) and C57/BL6 (Fig. 6A, B) mice. 2DG and 3NP-treated mice exhibited BACE1 increases of ~120-130% ($p < 0.05$) and ~160-170% ($p < 0.001$), respectively, in comparison to vehicle control treatment. Importantly, the BACE1 increases following chronic treatment were similar in magnitude to those observed under conditions of acute energy deprivation *in vivo* (Velliquette et al., 2005) and *in vitro* (this study).

Based on our *in vitro* studies with BACE1-293 cells and primary neurons, we predicted that the BACE1 increase following chronic *in vivo* energy deprivation would be post-transcriptional. To test this, we isolated mRNA from hemibrains of C57/BL6 mice chronically treated with 2DG, 3NP, and vehicle and measured levels of endogenous mouse BACE1 mRNA by TaqMan quantitative real-time PCR analysis. Indeed, BACE1 mRNA levels were not increased upon 2DG or 3NP treatment, and in fact were significantly *decreased* compared to vehicle (Fig. 6C). Although we do not fully understand the reason for this mRNA decrease, we speculate that it may be a result of global down-regulation of transcription caused by ATP depletion. In any case, our results clearly demonstrate that the BACE1 elevations in response to chronic *in vivo* energy deprivation were not the result of either increased BACE1 gene transcription or enhanced BACE1 mRNA stabilization.

Since our previous results showed that eIF2α phosphorylation and enhanced BACE1 translation were responsible for the energy deprivation-induced BACE1 increase *in vitro*, we wanted to determine whether increased eIF2α phosphorylation might account for the BACE1 elevation *in vivo*. To accomplish this, we used immunobot analysis to measure phosphorylated and total eIF2α levels in brain homogenates of Tg2576 mice treated with 2DG, 3NP, or vehicle (Fig. 5B, C). The eIF2α-P(Ser51):eIF2α-T ratios in the brains of 2DG and 3NP-treated mice were elevated to ~130% ($p < 0.05$) and ~150% ($p < 0.01$) of vehicle, respectively (Fig. 5C), similar to the increases in eIF2α phosphorylation observed *in vitro*. Importantly, mean increases in eIF2α phosphorylation correlated with the BACE1 elevations that occurred in 2DG and 3NP-treated mice. These results, together with our *in vitro* BACE1-293 cell and primary neuron data, suggest that chronic energy deprivation *in vivo* induces the eIF2α translational control pathway, thereby increasing BACE1 levels.

Previous studies had reported that modest overexpression of BACE1 increased Aβ levels and amyloid deposition in transgenic mice (Bodendorf et al., 2002; Chiocco et al., 2004; Chiocco and Lamb, 2007; Lee et al., 2005; Mohajeri et al., 2004; Ozmen et al., 2005; Willem et al., 2004). Therefore, the elevated BACE1 levels in 2DG and 3NP-treated Tg2576 mice suggested that levels of Aβ and amyloid plaques might also be increased in these animals. To investigate this, we measured $A\beta_{40}$ in brain homogenates by $A\beta_{40}$-specific ELISA and counted amyloid plaques in hemibrain sections from treated Tg2576 mice. 2DG and 3NP treatment caused clear trends toward increased $A\beta_{40}$ levels (Fig. 5D; VEH = 9.10 ± 1.30 ng $A\beta_{40}$/mg protein, 2DG = 13.15 ± 1.94, 3NP = 15.56 ± 3.75) and plaque numbers (Figs. 5E; VEH = 20.8 ± 3.7; 2DG = 26.7 ± 5.3; 3NP = 36.0 ± 8.6), with a nearly 2-fold increase occurring in 3NP-treated mice. Consistent with these observations, examination of brain sections stained with anti-Aβ antibody (Figs. 5F, S5) or Thioflavin-S (Fig. S5) showed that plaques in 2DG and 3NP-treated mice appeared larger and more numerous than in vehicle-treated mice. High inter-animal variability in treated mice caused the increases of $A\beta_{40}$ level and plaque number to not quite reach statistical significance, although it is likely that statistical significance would have been achieved with longer treatment times.

Importantly, we consistently observed that 3NP treatment always produced the strongest increases of phosphorylated eIF2α, BACE1, $A\beta_{40}$, and plaque number, while 2DG treatment always had more moderate effects (compare Figs. 5C through 5E). 3NP is a strong, irreversible inhibitor of glucose metabolism, in contrast to the weaker reversible inhibitor, 2DG. Thus, the effects of the drugs on phosphorylated eIF2α, BACE1, $A\beta_{40}$, and plaque number reflected their relative potency for inhibiting glucose metabolism. Taken together, these observations make a compelling case that chronic energy deprivation *in vivo* is likely to promote amyloidogenesis via a mechanism involving eIF2α phosphorylation and BACE1 translational control.

Finally, we wanted to exclude the possibility that other mechanisms were responsible for elevating BACE1 levels and promoting amyloidogenesis in 2DG and 3NP-treated Tg2576 mice, such as altered micro-RNA (miRNA) expression, increased γ-secretase, or decreased Aβ degrading enzyme levels. Recent studies have shown that miRNAs miR-29a, -29b-1, and -9 are decreased in AD and are capable of regulating BACE1 expression *in vitro* (Hebert et al., 2008; Wang et al., 2008). Therefore, we measured the levels of miR-29a, -29b-1, and -9 in treated Tg2576 mice by TaqMan quantitative RT-PCR analysis. We found that none of these miRNAs were decreased in brains subjected to chronic energy deprivation (Fig. S6). In fact, miR-29a and -29b-1 were *increased* in 3NP-treated mice, as compared to vehicle treatment, which would be expected to *decrease* BACE1 levels (Hebert et al., 2008; Wang et al., 2008). Next, we performed immunoblot analyses for the amino-terminal fragment (NTF) of presenilin 1 (PS1), a critical subunit of the γ-secretase, and insulin degrading enzyme (IDE) and neprilysin (NEP), two major Aβ degrading enzymes (Fig. S7). Overall, the levels of these molecules were not significantly affected by chronic energy deprivation *in vivo* (Fig. S7A, B), although a trend

toward elevated NEP levels was observed for 3NP-treated mice, a condition that would be expected to *decrease* Aβ levels. In addition, PS1-NTF, IDE, and NEP levels were not altered in Sal-treated Tg2576 primary neuron cultures (Fig. S7C, D). Taken together, these results make it unlikely that miRNAs, γ-secretase, or Aβ degrading enzymes played a major role in the increased levels of BACE1 and Aβ in response to chronic energy deprivation in Tg2576 mice.

Increased eIF2α Phosphorylation Correlates with Elevated BACE1 Levels in 5XFAD Transgenic Mice and Humans with AD

At this point in our analysis, the *in vitro* and *in vivo* energy deprivation experiments suggested the possibility that impaired energy metabolism in AD could increase BACE1 levels via a mechanism involving eIF2α phosphorylation and exacerbate amyloidogenesis. Reports had already shown that BACE1 levels and activity were increased in AD brains compared to non-demented controls (Fukumoto et al., 2002; Holsinger et al., 2002; Li et al., 2004; Sennvik et al., 2004; Tyler et al., 2002; Yang et al., 2003). Moreover, we had demonstrated that BACE1 levels were dramatically elevated around amyloid plaques in AD patients, Tg2576 mice, and in our aggressive amyloid deposition model, 5XFAD transgenic mice (Zhao et al., 2007). However, no study had yet addressed whether chronic energy deprivation or eIF2α phosphorylation was potentially involved with the BACE1 increase in AD. If impaired cerebral energy metabolism played a role in sporadic AD, and if the mechanism entailed increased translation of BACE1 as a result of eIF2α phosphorylation, then we would expect to observe increased levels of eIF2α-P and correlation between eIF2α-P and BACE1 levels in AD brain. Similar conditions might also exist in APP transgenic mice, since we observed a robust BACE1 increase in the brains of 5XFAD transgenic mice (Zhao et al., 2007).

In order to initially test these hypotheses, we analyzed levels of BACE1, eIF2α-P, and eIF2α-T by immunoblot in the brains of six-month-old 5XFAD mice that had florid amyloid pathology (Oakley et al., 2006). In agreement with our recent report (Zhao et al., 2007), we observed that BACE1 levels in 5XFAD brains were increased to ~170% of non-transgenic (non-Tg) littermate controls ($p < 0.01$; Fig. 7A, B). As we had predicted, levels of eIF2α-P(Ser51) were elevated in 5XFAD brain as well (Fig. 7A). Unexpectedly, however, total eIF2α levels were also increased in 5XFAD brain compared to non-Tg control. Importantly, in spite of the elevated eIF2α-T levels, the eIF2α-P(Ser51):eIF2α-T ratio was significantly increased to ~120% of non-Tg control in 5XFAD brains ($p < 0.05$; Fig. 7A, B). The mechanism and functional significance of the increase in total eIF2α remains unclear; however, we speculate that it may represent an adaptive response to chronic stress caused by the aggressive amyloid deposition in 5XFAD mice. It is noteworthy that increased BACE1 mRNA levels were not responsible for the BACE1 elevation in 5XFAD mice (Zhao et al., 2007), thus suggesting a post-transcriptional mechanism most likely involving eIF2α phosphrylation. In addition, we measured levels of miR-29a, -29b-1, and -9 and found no evidence that these miRNAs had altered expression in 5XFAD brains (Fig. S6). Finally, it is significant that 5XFAD mice had elevated cerebral eIF2α-P and BACE1 levels in the absence of pharmacologically induced energy deprivation, indicating that eIF2α phosphorylation could be increased by amyloid pathology as well as energy deprivation. This has important implications for a potential positive feedback mechanism that may drive BACE1 elevation and amyloidogenesis in AD (Fig. 9).

As the final step in our study, it was necessary to verify that eIF2α phosphorylation and BACE1 were elevated in AD brain. To accomplish this, we performed immunoblot analysis to measure levels of BACE1, eIF2α-P, and eIF2α-T in a series of cortical human brain samples from AD patients and age-matched, non-demented (N) controls (Fig. 7C, D). In agreement with previous reports, BACE1 levels were significantly elevated in AD brains (~140% of non-demented (N) control; $p < 0.01$). As predicted, AD brains also exhibited increased eIF2α-P(Ser51) levels

(Fig. 7C, D), and the AD eIF2α-P(Ser51):eIF2α-T ratio was found to be ~150% of N control ($p < 0.05$). We next performed linear regression analyses and determined that BACE1 level and amyloid load had a significant positive correlation in human brain, as expected ($p < 0.05$; Fig 7G). Importantly, BACE1 levels plotted against eIF2α-P(Ser51):eIF2α-T ratios also showed a significant positive correlation ($p = 0.0135$; Fig 7E), suggesting that the increase in eIF2α-P may cause the BACE1 elevation in human brain. Finally, amyloid load also exhibited significant positive correlation with eIF2α-P:eIF2α-T ratio ($p < 0.01$; Fig 7F), in further support of the hypothesis that eIF2α-P may promote amyloidosis. Taken as a whole, our 5XFAD mouse and human AD results strongly suggest that increased eIF2α phosphorylation plays a role in the elevation of BACE1 levels in the brain and contributes to the development of amyloid pathology.

Discussion

AD is a complex neurodegenerative disorder that may result from multiple environmental and genetic factors that affect both the production and clearance of Aβ in the brain, the interaction of Aβ with other proteins involved in AD pathogenesis, the susceptibility of neurons to Aβ toxicity, and the susceptibility of neural systems to functional impairment from neurodegeneration. Identification of the molecular mechanisms underlying these processes is essential for the development of rational therapeutics to treat or prevent this disorder. Numerous studies have linked impaired energy metabolism to AD (Craft and Watson, 2004; de Leon et al., 1983; Hoyer, 2004; Rapoport, 1999a, b; Steen et al., 2005). Studies of post-mortem AD brains have also shown increased BACE1 levels compared to age-matched, non-demented controls (Fukumoto et al., 2002; Holsinger et al., 2002; Li et al., 2004; Sennvik et al., 2004; Tyler et al., 2002; Yang et al., 2003). Our results indicate that these findings may be related. We showed previously that BACE1, the enzyme that initiates the amyloidogenic pathway, is up-regulated in response to acute energy deprivation *in vivo* (Velliquette et al., 2005). Here, we show that the energy deprivation-induced BACE1 increase 1) occurs via a translational control mechanism that is dependent upon the BACE1 mRNA 5'UTR, 2) is regulated by eIF2α phosphorylation, PERK kinase, and the UPR/ER stress response, and 3) raises Aβ production and promotes amyloid plaque formation. In addition, eIF2α phosphorylation positively correlates with increased BACE1 levels and amyloid loads in humans with AD and in 5XFAD transgenic mice. We conclude that age-related impairment of cerebral energy metabolism could induce eIF2α phosphorylation and increase BACE1 levels, thereby exacerbating amyloidogenesis in AD. Thus, our results may have important implications for both the initiation and progression of AD.

In sum, our experiments have excluded all possible mechanisms of the energy deprivation-induced BACE1 elevation, except for an increase in translation. Importantly, we found no evidence that the BACE1 increase involved BACE1 gene transcription. In fact, we observed *decreased* BACE1 mRNA levels in treated primary neuron cultures and mouse brains, clearly demonstrating a post-transcriptional mechanism. The absence of caspase 3 activation or BACE1 protein stabilization in our energy deprivation models indicates that the specific mechanism of increased BACE1 level described in our study is distinct from post-translational mechanisms controlling BACE1 protein levels (Tesco et al., 2007). Moreover, we have excluded a role for miRNAs, although altered expression of specific miRNAs may be an important post-transcriptional mechanism for the regulation of BACE1 levels (Hebert et al., 2008; Wang et al., 2008). Although our results indicate that the eIF2α-P pathway is the only post-transcriptional mechanism controlling energy deprivation-induced BACE1 elevation, which may be relevant to early amyloidogenesis in preclinical AD, in the later stages of the disease, it is possible that other BACE1-elevating mechanisms come into play, including microRNA dysregulation (Hebert et al., 2008; Wang et al., 2008), apoptosis (Tesco et al., 2007), and p25/cdk5 dysfunction (Cruz et al., 2006; Wen et al., 2008). These other mechanisms

may either act alone or in concert to increase BACE1 levels and accelerate Aβ production. Aβ overproduction, in turn, may propagate the stress response in the brain and exacerbate the progression of amyloid pathology by feeding back and further elevating eIF2α-P levels.

Although $A\beta_{40}$ levels and plaque numbers in 2DG and 3NP-treated Tg2576 mice did not quite reach a statistically significance increase, they clearly showed an increasing trend that matched the relative potency of each compound for inhibiting glucose metabolism (Fig. 5D, E). This potency effect also extended to the treatment-induced increases of BACE1 and eIF2a-P (Fig. 5D, E) and the decreases of BACE1 and APP mRNA levels (Fig. 6C) caused by the two drugs. Given how slowly plaques form in Tg2576, it is likely that a longer treatment period would have led to greater Aβ accumulation and subsequent statistical significance. Although complex and poorly understood, plaque formation appears to be governed by the balance between the production and degradation of Aβ in the brain. We excluded the possibility that increased amyloidogenesis in treated Tg2576 mice was a consequence of either increased PS1/γ-secretase or decreased Aβ degrading enzymes, IDE and NEP. On the contrary, the slight trend of increased NEP level in 3NP treated mice (Fig. S7B) may have partially limited the Aβ increase in these animals. Regardless, the fact that both plaque number and $A\beta_{40}$ level show the same relative increase as BACE1 and eIF2a-P in 2DG and 3NP-treated mice suggests that energy deprivation is amyloidogenic *in vivo*.

Translational Control of BACE1: the Potential Role of Cellular Stress

Many translationally regulated transcripts encode proteins that appear to be involved in cellular stress responses. Although the normal roles of APP and BACE1 are not well understood, one of the proposed functions of BACE1, APP, and APP cleavage fragments is that they may be involved in the response to stress or injury (Blasko et al., 2004; Kamenetz et al., 2003; Sambamurti et al., 2004; Tamagno et al., 2005; Tanahashi and Tabira, 2001; Tesco et al., 2007; Turner et al., 2003; Wen et al., 2004; Wen et al., 2008). One type of stress that appears to be capable of altering APP processing and elevating Aβ generation is impaired energy metabolism (Busciglio et al., 2002; Gasparini et al., 1997; Hoyer et al., 2005; Webster et al., 1998). Since BACE1 levels are increased by energy deprivation, our data lend further support to the hypothesis that BACE1 may play a normal role in neuronal stress-response.

Stress-induced signaling pathways are typically thought to activate downstream transcriptional targets; however, many of these pathways also converge upon various components of the cellular translation machinery (Holcik and Sonenberg, 2005; Pavitt, 2005; Schroder and Kaufman, 2006). It is becoming increasingly evident that translation is a highly regulated process that plays a crucial role in the cellular response to stress. The most tightly controlled step in translation under stress conditions is at the level of initiation. Phosphorylation of eIF2α at Ser51 is a major mechanism for regulation of translation initiation in response to various cellular stresses (reviewed in Clemens, 2001; Schroder and Kaufman, 2006).

eIF2α is known to be phosphorylated by at least four different kinases, including double-stranded RNA-activated protein kinase (PKR), PKR-like endoplasmic reticulum kinase (PERK), general control non-derepression 2 kinase (GCN2), and heme-regulated inhibitor (HRI), in response to a variety of stress stimuli, such as the unfolded protein response, amino acid deprivation, UV irradiation, heat shock, viral infection, and hypoxia (Fig. 8A, right). Exchange of GDP for GTP on eIF2, a requirement for formation of the eIF2·GTP·Met-$tRNA_i$ ternary complex, is catalyzed by eIF2B (Fig. 8A, left). Phosphorylation of eIF2α inhibits the dissociation of eIF2B from eIF2α, thereby preventing the exchange of GDP for GTP and reducing the rate of ternary complex formation (Pavitt, 2005;Proud, 2001;Schroder and Kaufman, 2006). Reduced availability of the ternary complex simultaneously lowers the rate of global protein synthesis and enhances the translation of mRNAs similar to BACE1 that are normally translated at low levels due to long, G-C rich, uORF-containing 5'UTRs (Fig. 8B,

right). Translational de-repression of these mRNAs can be mediated by phosphorylated eIF2α through a variety of different mechanisms that overcome 5' UTR mRNA secondary structure (Fig. S8B), inhibitory uORFs (Fig. S8A), or leaky ribosomal scanning (Pavitt, 2005;Schroder and Kaufman, 2006).

Recent studies have demonstrated that the 5' UTR of BACE1 mRNA inhibits translation, implying that BACE1 might be translationally regulated. However, the specific mechanism responsible for BACE1 translational repression has been a matter of debate. It has been suggested that the 5' UTR of BACE1 mRNA forms a translational barrier as the result of tightly folded secondary structure in the BACE1 5'UTR (Lammich et al., 2004). Alternatively, the three uORFs in the BACE1 mRNA 5'UTR may inhibit ribosomes from reaching the authentic BACE1 ORF (De Pietri Tonelli et al., 2004; Mihailovich et al., 2007). A related translational repression mechanism involving leaky scanning of ribosomes through the BACE1 mRNA 5' UTR and reinitiation at the BACE1 ORF has also been proposed (Zhou and Song, 2006). Finally, it has been suggested that ribosome shunting may occur, thus allowing ribosomes to bypass the BACE1 mRNA 5' UTR (Rogers et al., 2004), although this mechanism is rare.

Although these studies suggested that BACE1 translational control might play an important role in AD pathogenesis, it was still an open question whether BACE1 translational repression could be lifted under conditions of stress relevant to AD. Moreover, a potential role for the eIF2α-P pathway in the regulation of BACE1 translation had not been considered. Here, for the first time we provide evidence linking impaired energy metabolism, an AD-relevant stress, to BACE1 translational de-repression mediated by eIF2α phosphorylation. It is well established that phosphorylation of eIF2α can relieve translational repression caused by secondary structure or uORFs in the 5' UTR, the best studied example being that of the yeast GCN4 gene (Pavitt, 2005; Schroder and Kaufman, 2006). The reduced concentration of eIF2·GTP·Met-$tRNA_i$ ternary complex under conditions of high eIF2α-P allows 40S ribosomal subunits to scan along the 5' UTR for a longer period of time before reloading with ternary complex to form the 43S preinitiation complex. In this circumstance, 40S subunits are more likely to bypass secondary structure and uORFs in the 5' UTR, thereby increasing the probability that reloading will occur in close proximity to the major ORF and reinitiation of translation will happen at the authentic start codon (Fig. 8B, right). Our results showing that the energy deprivation-induced BACE1 increase occurs via a translational mechanism, and is dependent upon the BACE1 mRNA 5'UTR and involves eIF2α phosphorylation, as well as evidence that the BACE1 5'UTR has extensive secondary structure with uORFs that inhibit translation (Fig. S8; Lammich et al., 2004), are all consistent with a mechanism of eIF2α-P mediated translational de-repression under conditions of cellular stress (Fig. 8).

It is noteworthy that impaired energy metabolism in the form of glucose deprivation had the common effect of increasing levels of eIF2α-P and BACE1 in HEK-293 cells, primary neurons, and brains. The fact that different cell types responded similarly to energy deprivation suggests that a ubiquitous stress-induced pathway underlies the eIF2α-P increase. Our data indicate that PERK is likely to be the major, if not only, eIF2α kinase responsible for energy deprivation-induced BACE1 elevation, at least *in vitro*. Whether or not PERK is the eIF2α kinase responsible for the BACE1 increase in 5XFAD transgenic mouse and AD human brain remains to be determined. However, the involvement of PERK in energy deprivation-induced BACE1 elevation is completely consistent with the well-established role of PERK in the unfolded protein response (UPR), a ubiquitous protective mechanism found in all cells. PERK is activated by unfolded proteins in the ER, and consequent eIF2α phosphorylation concomitantly reduces global protein synthesis and increases mRNA translation for a specific subset of proteins that mediate the UPR. This protective mechanism prevents the accumulation of unfolded proteins that could threaten the survival of the cell. Clearly, glucose deprivation or other forms of deficient energy metabolism would reduce cellular concentrations of ATP,

impair protein folding in the ER, and induce the UPR, as has been previously shown (Lee, 2001). This scenario is likely given that protein folding in the ER is highly dependent upon ATP (Zhang and Kaufman, 2006). In addition, we speculate that neurotoxic Aβ oligomers or amyloid plaques may also activate the UPR, thus accounting for the eIF2α-P and BACE1 increases in 5XFAD mouse and human AD brains, at least in part.

Although our data favor PERK and the UPR, it is possible that another kinase may be involved in the energy deprivation-induced BACE1 elevation in the brain: GCN2, which is activated by amino acid deprivation in yeast and is the predominant eIF2α kinase expressed in the brain (Costa-Mattioli et al., 2005). Alternatively, other regulatory proteins may be altered in order to increase levels of eIF2α-P(Ser51), such as inhibition of constitutive repressor of eIF2α phosphorylation (CReP), which complexes with PP1 and is responsible for keeping eIF2α dephosphorylated under basal conditions (Jousse et al., 2003), or GADD34, which dephosphorylates eIF2α-P during the return to basal conditions following stress.

It is important to note that BACE1 is not expected to be the only target of the eIF2α translational control pathway induced by energy deprivation. Indeed, several mRNAs are translationally de-repressed by phosphorylated eIF2α, including activating transcription factor 4 (ATF4), CAAT/Enhancer binding protein (C/EBP), and cationic amino acid transporter-1 (Cat-1; Schroder and Kaufman, 2006). These molecules play beneficial roles in the response to cellular stress. Similarly, BACE1 may also perform a protective or repair function during acute neuronal stress or injury, such as re-myelination (Willem et al., 2006) or depressing excitotoxic synaptic activity (Kamenetz et al., 2003). Although eIF2α phosphorylation is expected to increase the translation of several stress-response proteins under the conditions of chronic energy deprivation, BACE1 is the only identified eIF2α target that is directly involved in amyloidogenesis and is therefore the most relevant to AD pathophysiology.

The role of eIF2α phosphorylation in the brain during normal and pathological physiology is not well understood. Studies using gene-targeted mice indicate that eIF2α phosphorylation plays a crucial role in normal neuronal function, especially in synaptic plasticity through the activation of GCN2 (Costa-Mattioli et al., 2005; Costa-Mattioli et al., 2007; Klann et al., 2004). During pathology, activation of the eIF2α pathway occurs in the brains of AD patients, APP transgenic mice, and mice undergoing pharmacologically induced seizures (this study; Carnevalli et al., 2006; Peel and Bredesen, 2003). In addition, eIF2α phosphorylation is also associated with risk factors for AD, such as atherosclerosis and traumatic brain injury (TBI; Schroder and Kaufman, 2006). Moreover, eIF2α phosphorylation and the UPR appear to be activated in several neurodegenerative diseases besides AD, including Parkinson's (PD), Huntington's, and amyotrophic lateral sclerosis (ALS; Malhotra and Kaufman, 2007; Paschen and Mengesdorf, 2005). AD and PD are often comorbid (Braak et al., 2005; Mitchell, 1999), and it is tempting to speculate they share an underlying primary abnormality that may involve eIF2α phosphorylation. Also, amyloid pathology and the risk of AD are increased in patients who have TBI or temporal lobe epilepsy (Gaitatzis et al., 2004; Mackenzie and Miller, 1994; Szczygielski et al., 2005), disorders that exhibit deficiencies in energy metabolism. Impaired glucose utilization is a well-established feature of AD, and AD risk factors may all have the common effect of reducing energy metabolism in the brain. It is interesting to note that impaired energy metabolism, which would be expected to activate the eIF2α pathway, appears to be a common factor in several neurodegenerative disorders. Neurodegeneration may result either directly or indirectly from energy deficits in specific neuronal populations, which in turn determine the pathology and symptoms of a particular disease (Beal, 1992; Soane et al., 2007). It is conceivable that energy deprivation and the eIF2α phosphorylation pathway could play a role in several neurodegenerative diseases, although BACE1 elevation and amyloidogenesis may not be significant in every case. Whether or not amyloid pathology is involved in a particular disease may depend on the specific neuronal population that is affected

by energy deprivation and the eIF2α pathway. In AD, amyloid plaques readily form in the cortex and hippocampus but not in the cerebellum, indicating that neuronal populations vary with respect to their propensity to form amyloid. While many neurodegenerative diseases may exhibit deficits in energy metabolism and activation of the eIF2α pathway, only a subset (e.g., AD, PD, TBI, epilepsy) may have amyloid pathology because of the specific nature of the neuron populations involved and how they respond to energy deprivation. Nevertheless, specifically for AD, our results strongly suggest that energy deprivation and the eIF2α phosphorylation pathway play major roles in the BACE1 increase observed in AD, and that this BACE1 elevation promotes Aβ production and amyloidogenesis.

Chronic Neuronal Stress, eIF2α, and Amyloid Pathology

Phosphorylation of eIF2α is usually observed as a transient event. In contrast, we found sustained elevations of phosphorylated eIF2α in our energy deprivation models. Likewise, the presence of elevated levels of eIF2α-P(Ser51) in the brains of 5XFAD mice and AD patients suggests that eIF2α can be chronically phosphorylated. It is unclear whether chronic eIF2α-P (Ser51) increases are important for cell survival under long-term stress. The fact that up to 36 hrs of glucose deprivation did not cause cell death *in vitro* and that 3 months of chronic energy deprivation did not induce neurodegeneration in our study implies that chronic elevations of eIF2α-P(Ser51) may be neuroprotective. eIF2α-P may have a dual function in the brain: 1) in response to acute stress, which affords the neuron the advantage of rapid production of stress-response proteins, and 2) in response to chronic stress, which may serve to down-regulate energy-expensive processes such as global transcription and translation, while producing stress-response proteins. This may enable the neuron to survive under long-term stress conditions. The ability to survive chronic stress may be particularly important in the brain, where neurons are not readily replaced. From this standpoint, Aβ accumulation and amyloid pathology can be viewed as eventual downstream consequences of chronic eIF2α-P(Ser51) and BACE1 elevations, which themselves may be serving protective functions, at least initially. However, over the decades that AD is likely to develop, BACE1 and amyloid build up to the point that Aβ-induced neurodegeneration begins. Interestingly, since various stresses are capable of elevating eIF2α-P levels, multiple types of chronic stress in the brain are potentially amyloidogenic. Importantly, elevated eIF2α-P(Ser51) levels in 5XFAD mouse and human AD brains suggest that chronic stress, BACE1, eIF2α-P(Ser51), and Aβ collaborate in a positive feedback loop in which amyloidogenesis becomes amplified once Aβ has accumulated to the level that it is capable of initiating and sustaining a stress response in the neuron (Fig. 9). This model also predicts that once amyloid pathology is initiated, it is likely to progress at an increasing rate, unless the amyloidogenic cycle is somehow interrupted. Thus, eIF2α phosphorylation may be involved in both the initiation and progression of sporadic AD. This hypothesis highlights the importance of developing new preventative and disease-modifying therapies for AD, especially those aimed at inhibiting the generation and accumulation of Aβ in the brain.

Materials and Methods

(See Supplementary Material for additional methods details)

Human Brain Tissue, Amyloid Loads, and Linear Correlations

Post-mortem frontal cortex tissues were obtained from AD (n = 9; 88.3 ± 4.1yrs) and normal (n = 13; 88.0 ± 4.8yrs) participants in the Rush Hospital Memory and Aging Project (R01AG17917; Bennett, David A; PI) following Rush University IRB approval. To determine amyloid load, brain sections (20 μm) were stained with anti-Aβ total antibody (MO0872; 1:100; Dako, Carpinteria, CA) via DAB immunohistochemistry, and amyloid staining was imaged and quantified with a stereological mapping station (Leica DMRBE microscope; computer

with StereoInvestigator software version 5.00; MicroBrightField Inc, Colchester, VT). A systematic random sampling scheme was used to capture video images of amyloid-stained sections for quantitative analysis of plaque deposition using a custom algorithm previously described (Bennett et al., 2004). The percent areas stained for each section were averaged, and those numbers were used for amyloid loads in statistical analyses. BACE1 and eIF2α-P levels in frontal cortex samples were measured by immunoblot analysis as described below and expressed as percent of the mean of the normal control group. Linear regressions and comparisons of means using the t-test were performed using GraphPad Prism and InStat software, respectively (GraphPad Software, Inc., San Diego, CA).

Animals and Drug Treatments

Tg2576 (Taconic, Hudson, NY) and C57/BL6 (NIA, Bethesda, MD) mice were administered single i.p. injections of 1x PBS (VEH), 1g/kg 2DG (Sigma, St. Louis, MO), or 80mg/kg 3NP (Sigma, St. Louis, MO) adjusted to pH 7.0, once a week beginning at 9 months of age (8-9 mice/group). At 12 months of age, mice were transcardially perfused, brains were excised and divided in half, and hemibrains analyzed by biochemistry or immunohistochemistry as described below. Tg6799 "5XFAD" male mice were bred in-house to B6/SJL F1 hybrid females (Taconic) and described previously (Oakley et al., 2006). All procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Northwestern University Animal Care and Use Committee.

Tissue Preparation for Biochemical Analysis

Hemibrains were flash frozen in liquid N_2 and stored at -80°C. Frozen mouse hemibrains or human cortical samples were homogenized in 1x PBS, 1% Triton-X 100, 1x protease inhibitor cocktail (Calbiochem, San Diego, CA), and 1x Halt phosphatase inhibitor cocktail (Pierce, Rockford, IL). Total protein concentration was determined by the BCA method (Pierce, Rockford, IL).

Human $A\beta_{40}$ ELISA

10 mg/ml of Tg2576 brain homogenate was extracted overnight in 5M guanidine-HCl at room temperature (RT). Brains were further diluted 225-fold in BSAT-DPBS with 1x protease inhibitor cocktail (Calbiochem, San Diego, CA), centrifuged at 16,000xg for 20 min. at 4°C, then diluted 2x with Aβ diluent with 1x AEBSF. Tg2576 cell media was diluted in Aβ diluent with 1x AEBSF. Total $A\beta_{40}$ levels in diluted samples were determined using a human $A\beta_{40}$-specific sandwich ELISA (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations.

Amyloid plaque counts

A set of eight evenly spaced 30 μm parasagittal sections were selected that spanned the entire medial-lateral dimension of each hemibrain from Tg2576 mice chronically treated with vehicle, 2DG, or 3NP. Sections were stained with anti-Aβ antibody 4G8, developed by DAB immunohistochemistry, counterstained with hematoxylin, and mounted as described below. The total number of amyloid plaques in the eight sections from each mouse was then visually counted at low magnification in a microscope and plaque number means, SEMs, and p-values calculated.

Immunoblot Analysis

Total protein concentrations in cell lysates or brain homogenates were determined by the BCA method (Pierce, Rockford, IL). 1-25 μg protein from brain homogenates or cell lysates were boiled in a 1:1 solution of SDS sample boiling buffer and 1x RIPA (cells) or homogenization buffer (brains) prior to being separated on 4-12% NuPAGE Bis-Tris gels in 1x MOPS running

buffer (Invitrogen, Carlsbad, CA) and transferred to Millipore Immobilon-P polyvinylidene difluoride (PVDF) membrane, as described previously (Zhao et al., 2007). Blots were blocked in 5% bovine serum albumin (BSA) in Tris-buffered saline, 0.1% Tween 20 (TBST; Sigma, St. Louis, MO) overnight, then incubated in primary antibody for 1 hr at RT or overnight at 4° C (a table of primary antibodies and dilutions is included in Supplementary Material). Blots were washed in TBST and incubated for 1hr in horseradish peroxidase (HRP)-conjugated secondary antibodies diluted 1:10,000 in 5% milk in TBST (Jackson ImmunoResearch Laboratories, West Grove, PA). Immunosignals were detected using enhanced chemiluminescence (ECL+, Amersham Biosciences, Piscataway, NJ, or SuperSignal West Femto, Pierce, Rockford, IL) and quantified using a Kodak CF440 imager (Rochester, NY). Densitometric analyses of immunoblots were performed using Kodak 1D 3.6 image analysis software. Immunosignals of phosphorylated proteins were normalized to their respective total proteins. For other proteins, immunosignals were normalized to β-actin immunosignal or were normalized per default by adding the same amount of total protein loaded per lane, as indicated in figure legends. Values were expressed as percentages of the mean of the control. 1μL of a 1:10 dilution of BACE1-293 cell lysate was used as a BACE1 positive control. 10μg $BACE1^{-/-}$ brain homogenate was used as a BACE1 negative control. ~10μg UV-treated 293 cell lysate was used as a positive control for eIF2α-P, c-Jun-P, and JNK-P (Cell Signaling Technology, Danvers, MA).

RNA isolation and real-time PCR, and microRNA analysis

Total RNA was isolated using RNeasy Mini kit (cells) or RNeasy Lipid Mini kit (brains; Qiagen, Valencia, CA). For real time PCR analysis, 1μg of total RNA from each sample was used for first-strand cDNA synthesis using SuperScript III (Invitrogen, Carlsbad, CA). 112.5ng cDNA from each sample was amplified *via* Real-Time PCR using Assays-on-Demand pre-mixed Taqman primer/probe set for mouse or human BACE1 and mouse APP mRNA, and normalized against 18s rRNA, using an 7900HT sequence analyzer (Applied Biosystems, Foster City, CA). mRNA levels for each experimental group were quantified using the comparative CT method. For microRNA analysis, RNA samples were analyzed using the Taqman microRNA reverse transcription kit with Taqman Universal PCR master mix (Applied Biosystems, Foster City, CA). Quantitative RT-PCR procedures were performed as previously described (Hebert et al., 2008) following manufacturer's recommendations (Applied Biosystems, Foster City, CA). Relative expression was calculated by using the comparative CT method.

Immunohistochemistry

Hemi-brains were drop-fixed in 4% paraformaldehyde, 1xPBS overnight and equilibrated in 30% sucrose in 1x PBS with 0.01% sodium azide at 4°C for at least 12 hrs for histology. 30μm frozen parasagittal sections from cryopreserved brains were cut using a sliding microtome and collected in Tris-buffered saline (TBS). Sections from Tg2576 brains were stained with Thioflavin S (for β-sheet amyloid) or incubated in anti-4G8 (1:5,000; Chemicon) overnight at room temperature, washed, and incubated with biotinylated secondary antibody (1:5,000; Chemicon) for 1 hr. The Vectorlabs ABC kit was used with DAB as chromagen to visualize the reaction product for 4G8, and sections were counterstained with hematoxylin. The total number of 4G8-positive plaques in each section was counted manually and average number of plaques per section was calculated (8-11 sections/mouse). Sections from C57/BL6 brains were stained with cresyl violet to verify that chronic energy inhibitor treatments did not cause neurodegeneration or neuron loss. Sections were mounted, coverslipped, and imaged using a Nikon Eclipse E800 microscope and Spot advanced digital camera (Diagnostic Instruments, Sterling Heights, MI).

Cell Line Cultures

pCMV-hBACE1 was created by subcloning BACE1 cDNA (Accession # AF190725) into the expression vector pCMVi (Cellular & Molecular Technologies, Inc., Lavallette, NJ). The BACE1-293 cell line was generated by co-transfecting HEK-293 cells with pCMV-hBACE1 and pRSV-Puro and selecting with 5μg/mL puromycin (Sigma, St. Louis, MO), and were maintained in selective DMEM (Invitrogen, Carlsbad, CA) containing D-glucose (4,500 mg/L). For energy deprivation experiments, cells were incubated in selective DMEM without glucose (Invitrogen, Carlsbad, CA). Transient transfections in BACE1-293 and HEK-293 cells were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). GADD34 ΔN, PERKDN, GCN2DN, and $P58^{IPK}$ vectors were gifts from David Ron (NYU). +5'UTR and -5'UTR BACE1 constructs were described previously (Lammich et al., 2004). Salubrinal (Calbiochem) was added to the cell media at 100μM for 24 hrs. Actinomycin D (Sigma, St. Louis, MO) was added to the cell media at 1.7μg/mL for 12 hrs. For taurodeoxycholate (TUDCA; Sigma, St. Louis, MO) experiments, cells were pre-treated with 1μM TUDCA (adjusted to pH 7.0) for 30 min., then treated for 12 hrs with glucose-deficient media containing 1μM TUDCA. For biochemical analyses, cells were lysed in 1x RIPA buffer with 1x protease inhibitor and 1x Halt phosphatase inhibitor cocktails, and protein content was measured using the BCA assay (Pierce, Rockford, IL).

Pulse-Chase

BACE1-293 cells were grown in 10-cm plates. Prior to pulse-labeling, cells were incubated in methionine/cysteine-deficient DMEM (MP Biochemicals, Solon, OH) for 1 hr. Cells were pulse-labeled with 2 mL of methionine/cysteine-deficient DMEM supplemented with 0.2mCi ^{35}S trans-labeled methionine and cysteine (MP Biochemicals, Solon, OH) for 20 min. Cells were washed 2x and "chased" with regular DMEM (Invitrogen, Carlsbad, CA). At 0, 3, 6, 12, and 24hrs post-label, lysates were homogenized in 1x RIPA and immunoprecipitated with Sepharose G beads (Amersham, Buckinghamshire, UK) and 3μg anti-BACE1 (PA1-757; Affinity Bioreagents, Golden, CO). Immunoprecipitates were separated using 10% Criterion Tris-HCl gels (BioRad; Hercules, CA). Gels were fixed, dried, and exposed to a phosphor screen overnight (Amersham, Buckinghamshire, UK). Autoradiographic signals were imaged using a Cyclone Plus Phosphor Imager (Perkin-Elmer, Waltham, MA).

Neuronal Cultures

Cortical tissue was isolated from E15.5-E16.5 C57/BL6 embryos, dissociated for 15 min. at 37°C in 5 ml of 0.25% trypsin (Invitrogen, Carlsbad, CA), washed with 1x BSS, and triturated. Neurons were plated for 2 hrs in serum-containing neurobasal media supplemented with B-27 (Invitrogen, Carlsbad, CA). Neurons were maintained for 2 DIV in neurobasal media without serum. At 2 DIV, neurons were switched to neurobasal media without glutamate, after which half of the maintenance media was changed every 2-3 days. Treatments began at 7 DIV. For energy deprivation experiments, neurons were treated for 36 hrs in Locke's solution with or without 20mM glucose (Cheng and Mattson, 1995). For pharmacological treatments, salubrinal was added to the media at 50μM or 80μM for 48 hrs, and rapamycin (Cell Signaling) was added to the maintenance media at 10nM for 12 hrs. For biochemical analyses, media and cell lysates were collected, and lysates were analyzed for total protein content using the BCA assay.

Statistical analysis

Data are presented as means ± standard errors of the mean (SEMs, represented by error bars in histograms). N-values are stated in figure legends. Statistical significance between means of experimental and control groups was determined using the two-tailed t-test (*$p < 0.05$, **p 0.01, ***$p < 0.001$).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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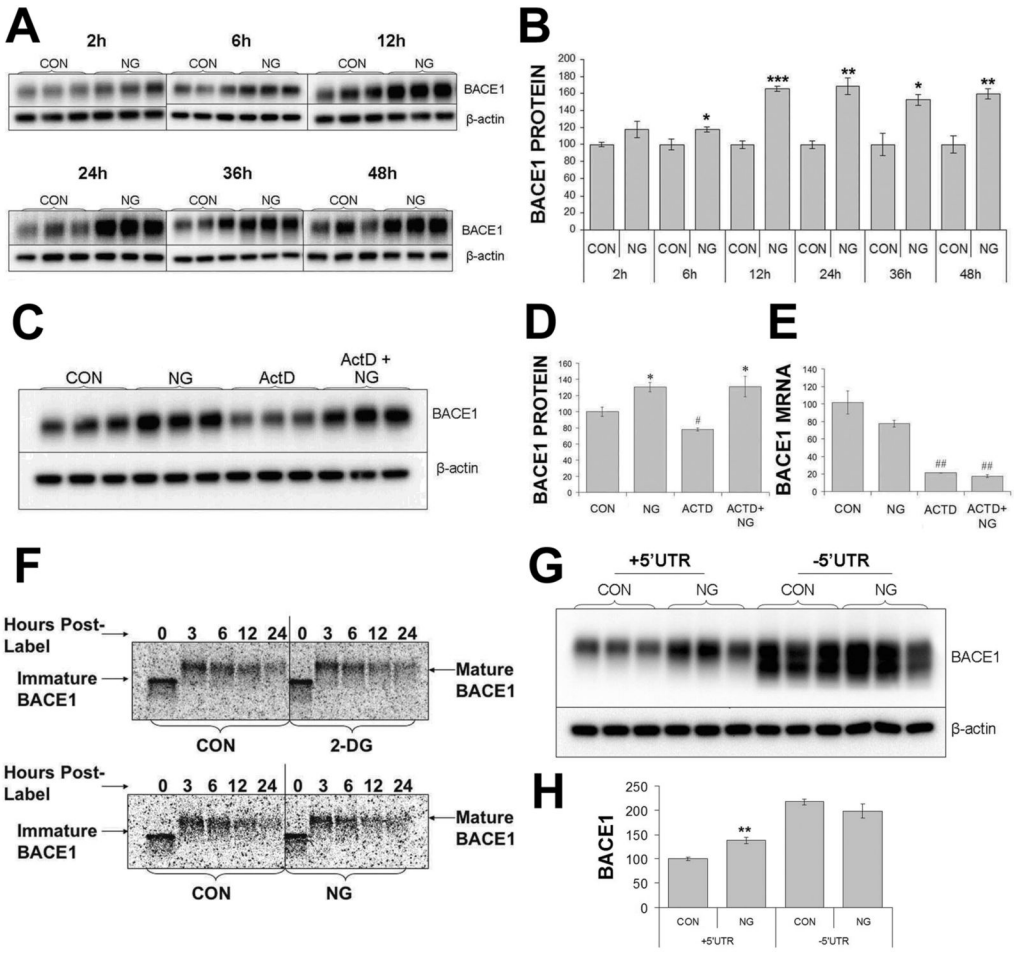

Figure 1. Glucose deprivation increases BACE1 protein levels in BACE1-293 cells *via* a post-transcriptional mechanism

(A) BACE1-293 cells were incubated for 3, 6, 12, 24, 36, and 48 hrs in either normal DMEM with glucose (25mM; CON) or DMEM without glucose (NG). Cells were lysed at the end of the treatment periods and 5μg of total protein per lane was used for immunoblot analysis of BACE1. β-actin was used as a loading control. **(B)** BACE1 immunosignals in (A) were quantified by phosphorimager, normalized to β-actin, and expressed as percentage of control (CON) for each time-point. BACE1 protein levels were significantly elevated compared to CON in response to glucose deprivation from 6 through 48 hrs (mean ± SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). An upward trend for a BACE1 increase was present at 2 hrs of NG treatment (n=3 per group). **(C)** Triplicate wells of BACE1-293 cells were incubated for 12 hrs in regular DMEM with glucose (CON), DMEM without glucose (NG), DMEM with glucose containing 1.7μg/mL actinomycin D (ActD), or DMEM without glucose containing 1.7μg/mL actinomycin D (ActD + NG). Cells were lysed at the end of the treatment period and 5μg of total protein was used for immunoblot analysis of BACE1. β-actin was used as a loading control. **(D)** BACE1 immunosignals in (C) were quantified by phosphorimager, normalized to β-actin, and expressed as percentage of control (CON). BACE1 protein levels were significantly elevated in response to 12 hrs of NG treatment either in the presence or absence of ActD, compared to CON (*, $p<0.05$), demonstrating that BACE1 gene transcription was not required for the BACE1 increase. BACE1 protein levels were significantly decreased with 12 hrs of ActD treatment alone, compared to CON (mean ± SEM; #, $p<0.05$; n=3 per group)

(E) Parallel BACE1-293 cell cultures were treated as in (C), except that total mRNA was isolated and levels of BACE1 mRNA measured via the TaqMan real-time PCR relative quantification method and expressed as percentage of control (CON; n=6). There were no differences in BACE1 mRNA levels between cells treated in media with or without glucose, verifying that NG treatment did not increase BACE1 gene transcription or mRNA stability. Note that ActD performed as expected and produced a robust decrease of BACE1 mRNA in cells treated with or without glucose, compared to CON (mean ± SEM; ##, $p<0.01$). **(F)** BACE1-293 cells were pulse-labeled in media containing ^{35}S-methionine/cytseine and chased for up to 24 hrs in normal DMEM with glucose (CON), DMEM without glucose (NG), or DMEM containing 50mM 2-deoxyglucose (2-DG). Radiolabeled BACE1 was immunoprecipitated from cell lysates at 0, 3, 6, 12, and 24 hrs post-label, separated *via* SDS-PAGE, and visualized by autoradiography. Note that immaturely glycosylated BACE1 is ~50kDa, while maturely glycosylated BACE1 is ~70 kDa. There were no apparent differences between the half-lives of BACE1 protein under CON, NG, and 2DG conditions, indicating that BACE1 increases are not due to BACE1 protein stabilization. In histograms, error bars represent S.E.M. **(G)** HEK-293 cells were transiently transfected with pcDNA3.1Zeo(+) vector containing the entire human BACE1 coding region (~1.5kb) plus the BACE1 5'UTR (+5'UTR) or pcDNA3.1Zeo(+) vector containing only the human BACE1 coding region (-5'UTR; (Lammich et al., 2004). Following overnight recovery, cells were incubated for 24 hrs in normal DMEM with glucose (CON) or DMEM without glucose (NG). Cell lysates were prepared and 5μg of total protein per lane was used for immunoblot analysis of BACE1 and β-actin. Note that transfection with the -5'UTR construct caused the accumulation of immature ~60kDa BACE1, in addition to mature ~70kDa BACE1 (Haniu et al., 2000). **(H)** BACE1 immunosignals in (G) were normalized to β-actin and expressed as percentage of +5'UTR control (CON, +5'UTR). The glucose deprivation-induced BACE1 increase did not occur in the absence of the BACE1 5'UTR, implicating a BACE1 5'UTR-dependent translational control mechanism. (n=3 per group; **, comparison between CON and NG, +5'UTR; $p < 0.01$; mean ± SEM). Error bars = S.E.M. in all histograms.

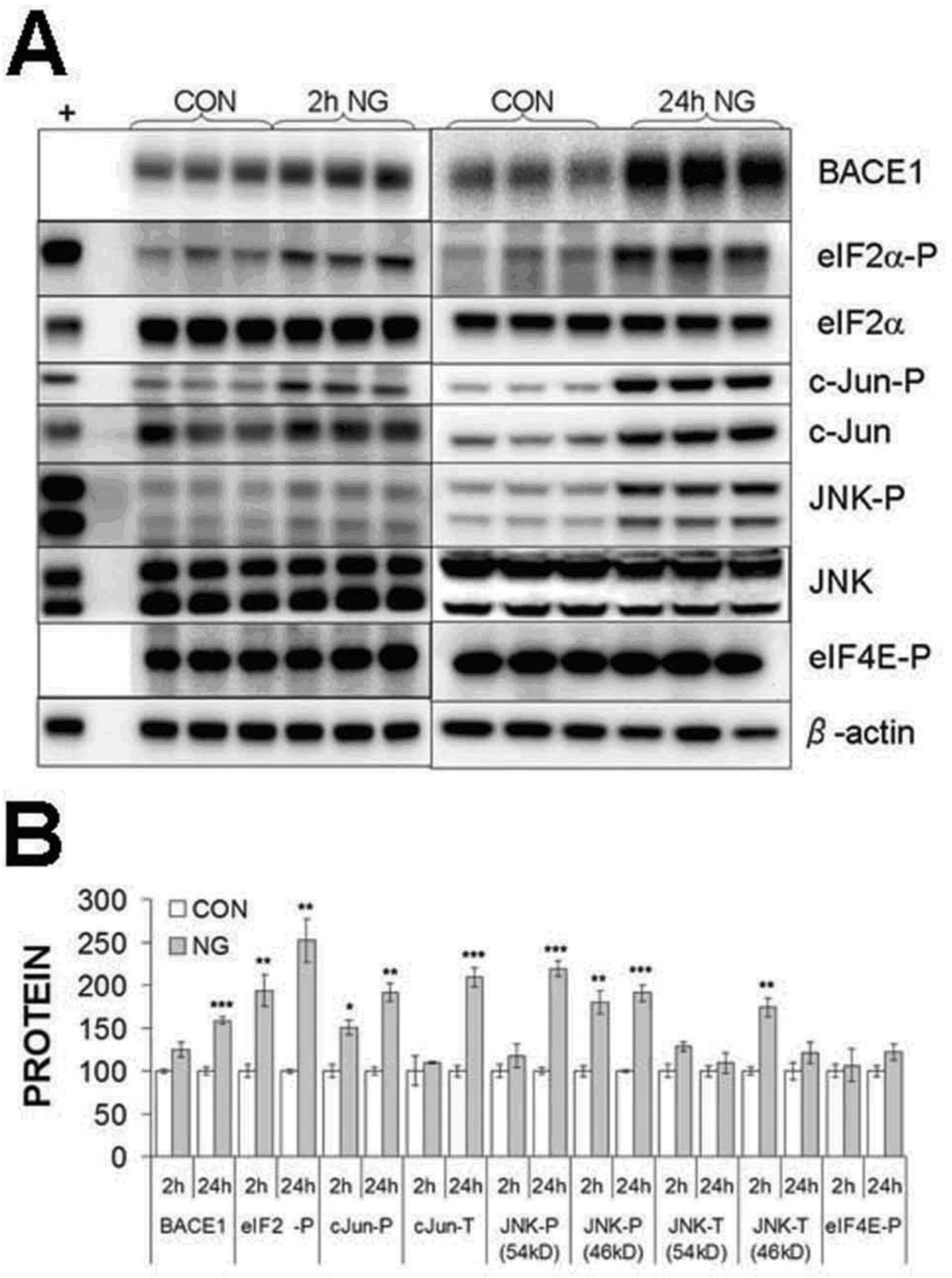

Figure 2. Glucose deprivation increases eIF2α phosphorylation and activates a specific set of stress-response signaling pathways in BACE1-293 cells

(A) BACE1-293 cells were incubated for 2 or 24 hrs in either normal DMEM with glucose (4,500 mg/L; CON) or DMEM without glucose (NG; n=3 wells per group). Cells were lysed and 10μg of total protein per lane was used for immunoblot analysis of BACE1, phospho-eIF2α (Ser51), total eIF2α, phospho-c-Jun(Ser63), total c-Jun, phospho-JNK(Thr183/Thr185), total JNK (54kD and 46kD isoforms), phospho-eIF4E(Ser209), and β-actin (see Supplement for list of antibodies used). ~10μg of lysate from UV-treated 293 cells was used as a positive control (+) for induction of stress-response pathways. **(B)** Immunosignals in (A) were quantified by phosphorimager and normalized in the following ways: BACE1, eIF2α, JNK,

and eIF4E-P were normalized to β-actin; eIF2α-P was normalized to total eIF2α ; c-Jun-P was normalized to c-Jun, and JNK-P was normalized to JNK. Values are expressed as percentage of control (CON) for each time-point (error bars = S.E.M.). After 2 hrs of NG treatment, eIF2α-P, c-Jun-P, JNK-P(46kD), and JNK(46kD) were all significantly elevated. At 24 hrs NG, eIF2α-P, c-Jun-P, and JNK-P(46kD) remained elevated, and BACE1, c-Jun, and JNK-P (54kD) became significantly increased as well. eIF4E phosphorylation was unaffected by NG treatments at either time-point. These results show that eIF2α-P is temporally increased before BACE1, as expected if eIF2α-P regulates BACE1 translation. (mean ± SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

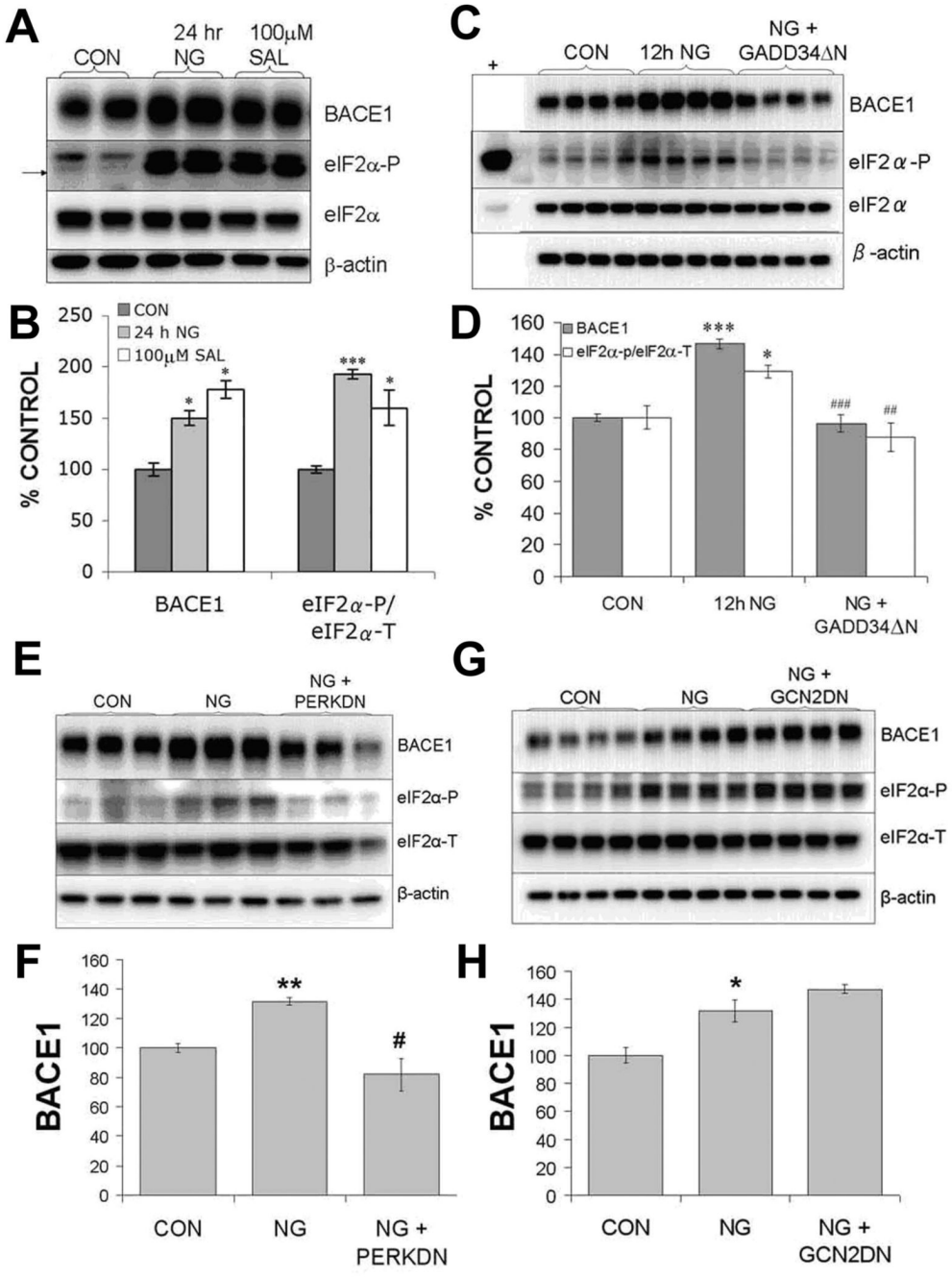

Figure 3. Direct manipulation of eIF2α phosphorylation affects the BACE1 increase in BACE1-293 cells

(A) BACE1-293 cells were incubated for 24 hrs in normal DMEM with glucose (25mM; CON), DMEM without glucose (NG), or DMEM containing 100μM salubrinal (SAL). Cell lysates were prepared and 10μg of total protein per lane was used for immunoblot analysis of phosphorylated eIF2α and total eIF2α. A parallel immunoblot was prepared with 5μg of cell lysate per lane for analysis of BACE1 and β-actin. The arrow identifies the authentic eIF2α-P band (the upper band is non-specific). **(B)** Immunosignals in (A) were quantified by phosphoimager, normalized, and expressed as percentage of control (CON). BACE1 immunosignals were normalized to β-actin, and eIF2α-P was normalized to total (T) eIF2α.

Levels of BACE1 and eIF2α-P/eIF2α-T were significantly elevated in response to both glucose deprivation and salubrinal treatment, compared to control (mean ± SEM; *, $p<0.05$; ***, $p<0.001$; n = 2 per group). Note that salubrinal treatment caused direct increases of eIF2α-P and BACE1 levels in the absence of glucose deprivation. These salubrinal-induced increases were similar in size to those caused by no-glucose treatment. **(C)** BACE1-293 cells were transiently transfected with GADD34 control vector (CON, 12h NG) or GADD34ΔN (NG + GADD34ΔN), the constitutively active PP1 regulatory subunit. Following overnight recovery, cells were incubated for 12 hrs in normal DMEM with glucose (CON) or DMEM without glucose (12h NG, NG + GADD34ΔN). Cell lysates were prepared and 10μg of total protein per lane was used for immunoblot analysis of BACE1, P-eIF2α (Ser51), total eIF2α, and β-actin. "+" lane: 10μg of lysate from UV-treated 293 cells was used as a positive control for eIF2α-P. **(D)** Immunosignals in (C) were quantified by phosphoimager, normalized, and expressed as percentage of control (CON). BACE1 immunosignals were normalized to β-actin, and eIF2α-P was normalized to total eIF2α. Glucose deprivation-induced increases in levels of BACE1 and eIF2α-P/eIF2α-T were completely prevented by overexpression of GADD34ΔN, demonstrating that eIF2α phosphorylation was responsible for the BACE1 increase. (n=4; comparison between CON and 12h NG: *, $p < 0.05$; ***, $p < 0.001$; comparison between 12h NG and NG + GADD34ΔN: ##, $p < 0.01$; ###, $p < 0.001$; mean ± SEM). **(E)** BACE1-293 cells were transiently transfected with pcDNA3 empty vector (CON, NG) or dominant negative PERK (PERKDN). Following overnight recovery, cells were incubated for 24 hrs in normal DMEM with glucose (CON) or DMEM without glucose (NG, NG + PERKDN). Cell lysates were prepared and 10μg of total protein per lane was used for immunoblot analysis of phosphorylated eIF2α and total eIF2α. A parallel immunoblot was prepared with 5μg of cell lysate per lane for immunoblot analysis of BACE1 and β-actin. **(F)** BACE1 immunosignals in (E) were quantified by phosphoimager and expressed as percentage of control (CON). Glucose deprivation-induced increases in BACE1 levels were completely prevented by overexpression of PERKDN, demonstrating that PERK was the eIF2α kinase responsible for eIF2α phosphorylation and the BACE1 increase. (n=3 per group; **, comparison between CON and NG, $p < 0.01$; $^{\#}$, comparison between NG and NG + PERKDN, $p < 0.05$; mean ± SEM). **(G)** BACE1-293 cells were transiently transfected with pcDNA3 empty vector (CON, NG) or dominant negative GCN2 (GCN2DN). Following overnight recovery, cells were incubated for 24 hrs in normal DMEM with glucose (CON) or DMEM without glucose (NG, NG + GCN2DN). Cell lysates were prepared and 10μg of total protein per lane was used for immunoblot analysis of phosphorylated eIF2α and total eIF2α. A parallel immunoblot was prepared with 5μg cell lysate per lane was used for immunoblot analysis of BACE1 and β-actin. **(H)** BACE1 immunosignals in (G) were quantified by phosphoimager and expressed as percentage of control (CON). The glucose deprivation-induced increase in BACE1 level was not prevented by overexpression of GCN2DN, demonstrating that GCN2 was not the eIF2α kinase responsible for eIF2α phosphorylation and the BACE1 increase. (n=3 per group; *, comparison between CON and NG, $p < 0.05$; mean ± SEM). Error bars = S.E.M. in all histograms.

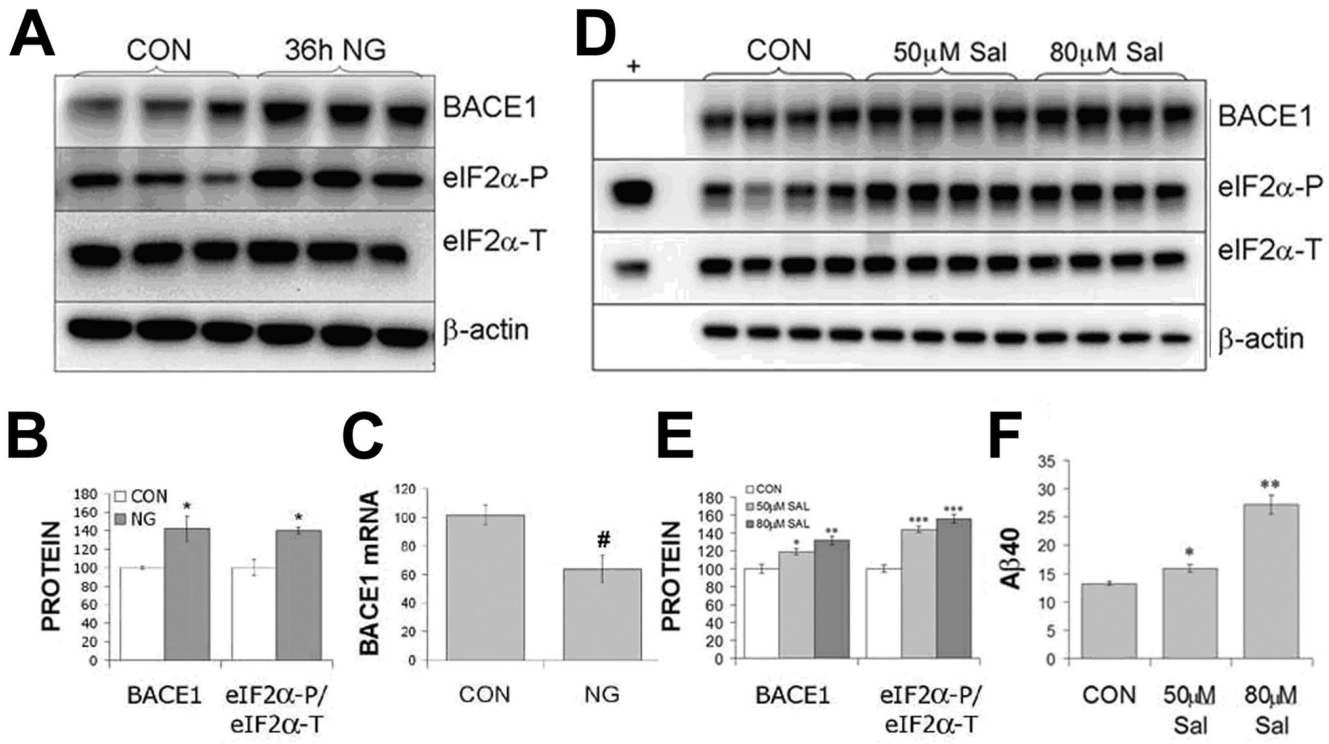

Figure 4. eIF2α phosphorylation causes post-transcriptional increases of BACE1 and promotes Aβ production in cultured primary neurons

(A) Primary cortical C57/BL6 neurons were cultured for 7 DIV and then incubated for 36 hrs in media containing 20mM glucose (CON) or no glucose (NG). Cell lysates were prepared and 10μg of total protein per lane was used for immunoblot analysis of BACE1, P-eIF2α(Ser51), total eIF2α (eIF2α-T), and β-actin. **(B)** Immunosignals in (A) were quantified by phosphorimager, normalized, and expressed as percentage of control (CON). BACE1 immunosignals were normalized to β-actin, and eIF2α-P was normalized to eIF2α-T. Levels of endogenous neuronal BACE1 and eIF2α-P/eIF2α-T were significantly elevated in response to glucose deprivation (mean ± SEM; *, $p < 0.05$; n=3), and the sizes of the increases were similar to those observed in BACE1-293 cells. **(C)** Parallel C57/BL6 primary neuron cultures were treated as in (A), except that total mRNA was isolated and levels of endogenous BACE1 mRNA measured via the TaqMan real-time PCR relative quantification method and expressed as percentage of control (CON; n=3). Unexpectedly, neuronal BACE1 mRNA levels were significantly reduced in cultures treated with NG-media compared to glucose-containing media (CON) (#; $p < 0.05$; mean ± SEM), clearly demonstrating a post-transcriptional mechanism for the BACE1 protein increase. **(D)** Primary cortical Tg2576 neurons were cultured for 7 DIV and then incubated for 48 hrs in normal media with glucose (CON), glucose-containing media with 50μM salubrinal (Sal), or glucose-containing media with 80μM Sal. Cell lysates were prepared and 10μg of total protein per lane was used for immunoblot analysis of BACE1, phosphorylated eIF2α, total eIF2α, and β-actin. 10μg of lysate from UV-treated 293 cells was used as a positive control (+) for eIF2α-P. **(E)** Immunosignals in (D) were quantified by phosphorimager, normalized, and expressed as percentage of control (CON). BACE1 was normalized to β-actin, and eIF2α-P was normalized to eIF2α-T. BACE1 levels and eIF2α-P/eIF2α-T ratios were significantly increased in Tg2576 neurons treated with both concentrations of Sal compared to control (CON; n=4; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; mean ± SEM), demonstrating that direct induction of eIF2α phosphorylation causes BACE1 levels to rise. **(F)** Conditioned media collected from 48hr salubrinal-treated Tg2576 neurons in (D) were

analyzed using a human $A\beta_{40}$ ELISA. Values are expressed as ng of $A\beta_{40}$ in the media per mg of total protein in the corresponding cell lysate. $A\beta_{40}$ levels were significantly elevated in Tg2576 neurons treated with both 50μM and 80μM salubrinal compared to control (CON; n=4; *, $p<0.05$; **, $p<0.01$; mean ± SEM), demonstrating that direct induction of eIF2α phosphorylation led to an increase in Aβ production.

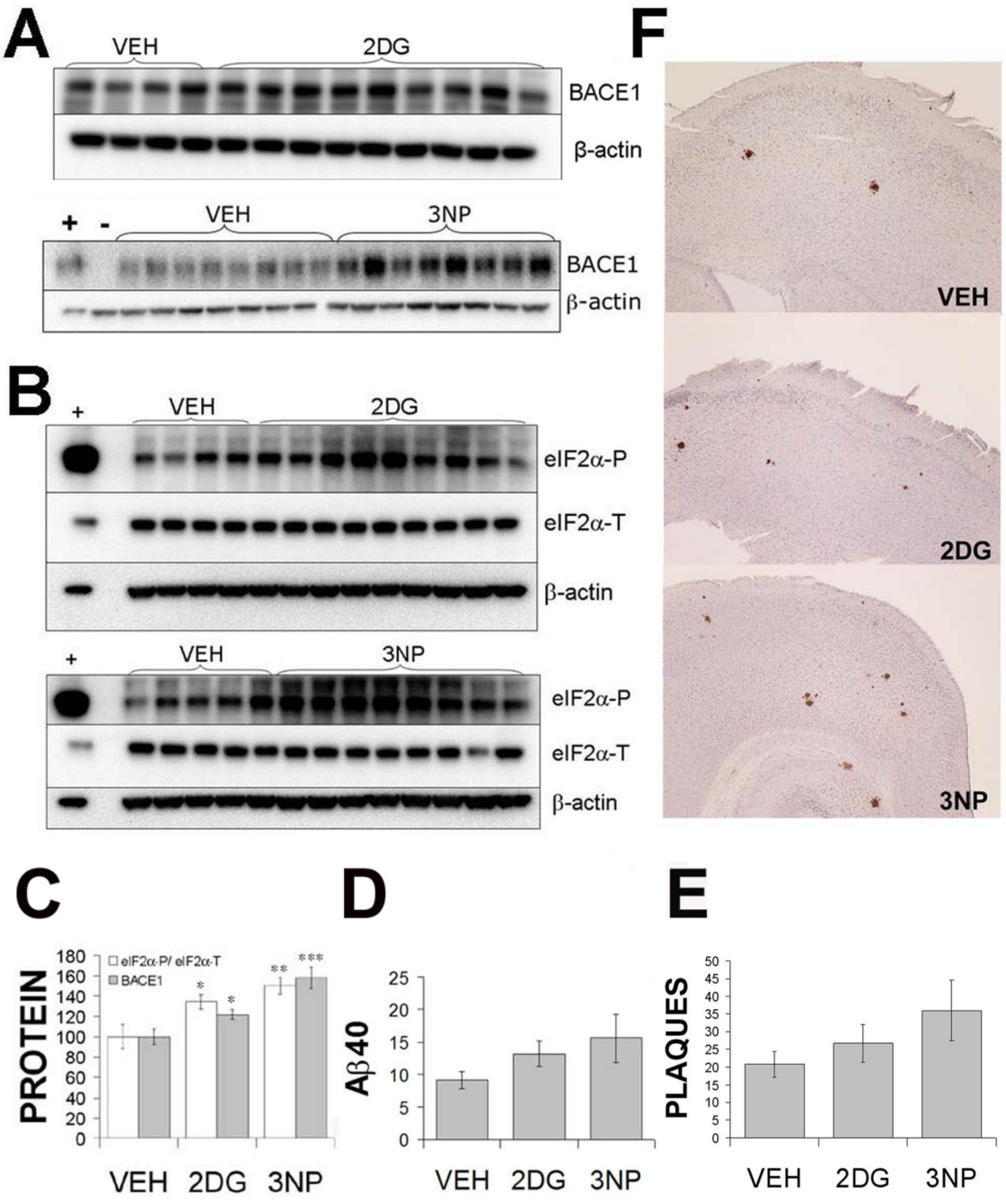

Figure 5. Elevated BACE1 levels are correlated with increased eIF2α-P and amyloidogenesis in a chronic model of energy deprivation *in vivo*

(A) Nine-month old Tg2576 mice were administered i.p. injections of saline (VEH), 1g/kg 2-deoxyglucose (2DG), or 80mg/kg 3-nitropropionic acid (3NP) once a week for 3 months (8-9 mice/group). One week after the final injection, hemibrains from each mouse were harvested and prepared for immunoblot and immunohistochemical analyses. Brain homogenates were prepared and 10μg of total protein per lane were used for immunoblot analysis of total BACE1 protein and β-actin. "+" lane: 5μg of BACE1-293 cell lysate as a BACE1 positive control.. "-" lane: 10μg of BACE1$^{-/-}$ mouse brain homogenate as a BACE1 negative control. **(B)** 15μg of total protein per lane of brain homogenate from VEH, 2DG, 3NP treated Tg2576 mice were

used for immunoblot analysis of eIF2α-P, eIF2α-T, and β-actin. "+" lane: 10μg of lysate from UV-treated 293 cells as a positive control for eIF2α-P. **(C)** Immunosignals in (A) and (B) were quantified by phosphorimager and expressed as percentage of vehicle (VEH). BACE1 levels and eIF2α-P/eIF2α-T ratios were significantly elevated in 2DG and 3NP treated Tg2576 mice compared to VEH (mean ± SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). **(D)** Guanidine-extracted brain homogenates from VEH, 2DG, and 3NP treated Tg2576 mice were analyzed using a human $A\beta_{40}$ ELISA. Values were expressed as ng of $A\beta_{40}$ per mg of total protein. A clear trend toward elevation of $A\beta_{40}$ level was observed in 2DG and 3NP treated mice, although values did not reach statistical significance. Increases in $A\beta_{40}$ levels tended to parallel the elevations of eIF2α-P and BACE1 for 2DG and 3NP treatments (compare C and D). **(E)** Fixed hemibrains of each mouse treated with VEH, 2DG, and 3NP were sectioned and stained with anti-Aβ antibody 4G8. The total number of 4G8-immunopositive plaques was counted in a set of eight evenly spaced parasagittal sections that spanned the entire medial-lateral dimension of each hemibrain. A clear trend toward an increase in plaque number was observed for both 2DG and 3NP treatments compared to VEH. Plaque numbers for the different treatments paralleled the relative levels of $A\beta_{40}$, as expected (compare to 5D), as well as the levels of eIF2α-P and BACE1 (compare to 5C). **(F)** Representative parasagittal brain sections from VEH, 2DG, and 3NP treated Tg2576 mice were immunostained with 4G8 and micrographed at 4x. 4G8-positive plaques in the brains of 2DG and 3NP treated mice tended to be larger and more numerous than those in VEH treated mice.

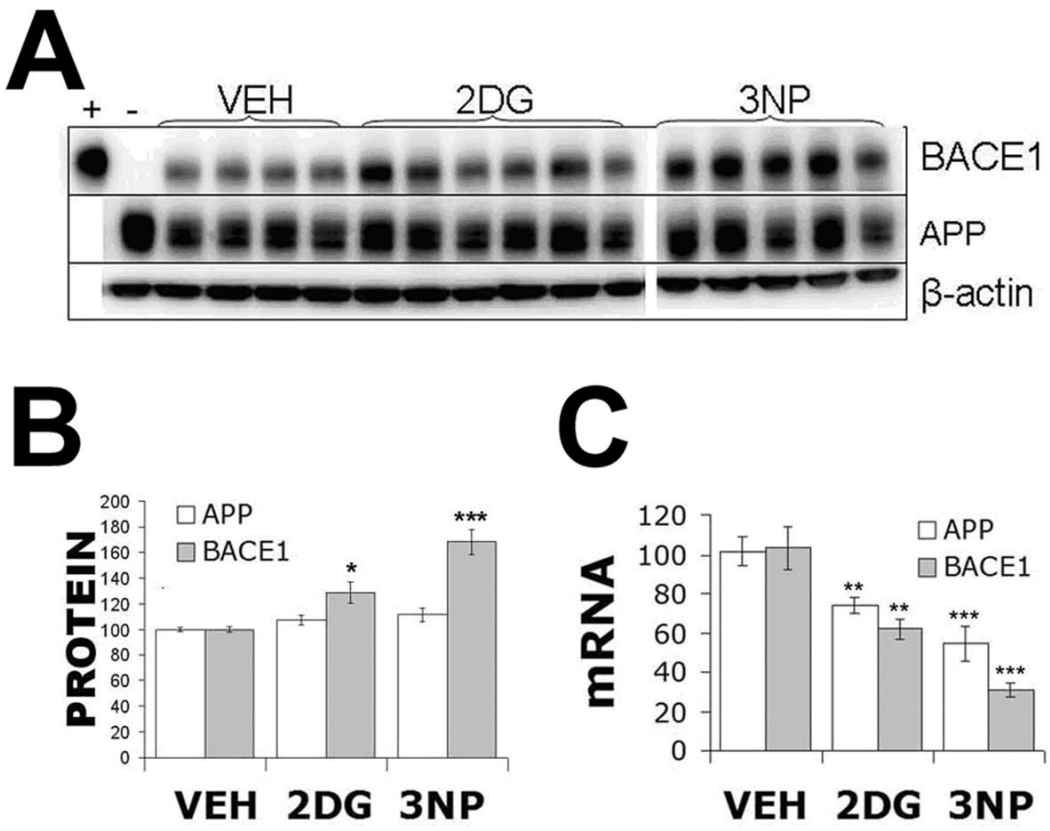

Figure 6. Chronic energy deprivation causes post-transcriptional increases of BACE1 *in vivo*

(A) Nine-month old wild-type C57/BL6 mice were administered i.p. injections of saline (VEH), 1g/kg 2-deoxyglucose (2DG), or 80mg/kg 3-nitropropionic acid (3NP) once a week for 3 months. One week after the final injection, hemibrains from each mouse were harvested and prepared for immunoblot and TaqMan quantitative real-time PCR analyses. Brain homogenates were prepared and 10μg of total protein per lane were used for immunoblot analysis of BACE1, full-length APP, and β-actin. "+" lane: 5μg of BACE1-293 cell lysate as a BACE1 positive control. "-" lane: 10μg of $BACE1^{-/-}$ mouse brain homogenate as a BACE1 negative control. **(B)** Immunosignals in (A) were quantified by phosphorimager and expressed as percentage of vehicle (VEH; n = 4-6 per group). BACE1 levels were significantly elevated in the brains of both 2DG and 3NP treated C57/BL6 mice compared to VEH (mean ± SEM; *, $p<0.05$; ***, $p<0.001$). **(C)** Total mRNA was isolated from the hemibrains of VEH, 2DG, and 3NP treated C57/BL6 mice and levels of endogenous BACE1 and APP mRNAs measured via the TaqMan real-time PCR relative quantification method and expressed as percentage of vehicle (VEH; 9-12 mice per group). Strikingly, both BACE1 and APP mRNA levels were significantly decreased in 2DG and 3NP treated C57/BL6 mice compared to VEH (mean ± SEM; **, $p<0.01$; ***, $p<0.001$).

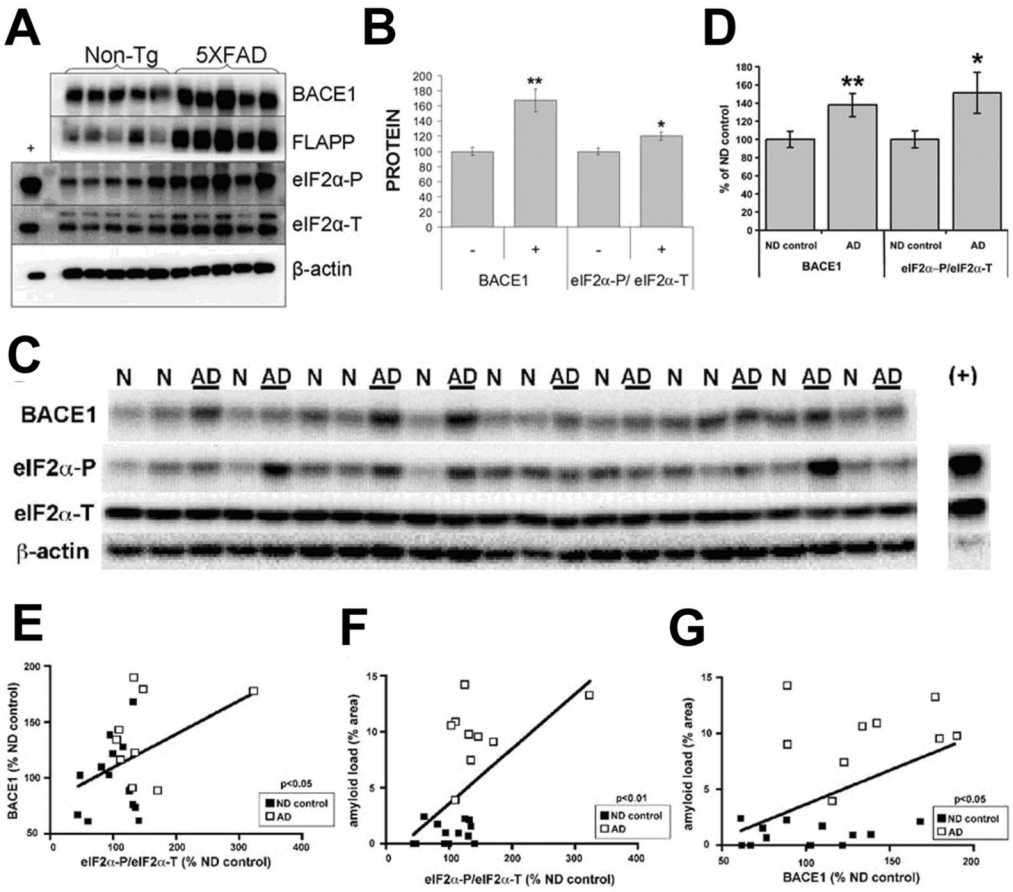

Figure 7. eIF2α-P levels are elevated in 5XFAD transgenic mouse and human AD brains and positively correlate with BACE1 levels and amyloid loads

(A) Brain homogenates were prepared from 6-month old 5XFAD transgenic (5XFAD) mice and non-transgenic littermates (Non-Tg), and 10μg of total protein per lane was used for immunoblot analysis of BACE1, full-length APP (FLAPP), eIF2α-P, eIF2α-T, and β-actin. "+" lane: 10μg of lysate from UV-treated 293 cells was used as a positive control for eIF2α-P. **(B)** Immunosignals in (A) were quantified by phosphorimager, normalized, and expressed as percentage of non-transgenic littermate levels. BACE1 and eIF2α-T immunosignals were normalized to β-actin, and eIF2α-P was normalized to eIF2α-T. "-": non-transgenic littermates; "+": 5XFAD transgenic mice. BACE1 levels and eIF2α-P/eIF2α-T ratios were significantly elevated in 5XFAD mice compared to Non-Tg (n=5 mice/group; *, $p<0.05$; **, $p<0.01$; mean ± SEM). **(C)** Human post-mortem frontal cortex samples from AD patients (AD) and age-matched non-demented controls (N) were homogenized and prepared for immunoblot analysis as described (Materials and Methods). 15μg of total protein per lane were analyzed by immunoblot for BACE1, eIF2α-P, eIF2α-T, and β-actin. 10μg of lysate from UV-treated 293 cells was used as a positive control for eIF2α-P (+). **(D)** Immunosignals in (C) were quantified by phosphorimager, normalized, and expressed as percentage of non-demented (ND) control. BACE1 and eIF2α-T immunosignals were normalized to β-actin, and eIF2α-P was normalized to eIF2α-T. Both BACE1 level and eIF2α-P/eIF2α-T ratio were significantly elevated in AD brains compared to ND (n=9 AD, 13 ND; *, $p<0.05$; **, $p<0.01$; mean ± SEM). **(E-G)** BACE1 levels and eIF2α-P/eIF2α-T ratios determined in (D) and amyoid loads (% area; Materials and Methods) were correlated by linear regression analyses of BACE1 level vs. eIF2α-P/eIF2α-T ratio (E), amyloid load vs. eIF2α-P/eIF2α-T ratio (F), and amyloid load vs. BACE1 level (G) for individual AD and ND frontal cortex samples. Amyloid load was expressed as the average

percent area of AD or ND frontal cortex section occupied by plaques immunostained with total anti-Aβ antibody. Statistically significant correlations were found for BACE1 level vs. eIF2α-P/eIF2α-T ratio ($p < 0.05$), amyloid load vs. eIF2α-P/eIF2α-T ratio ($p < 0.01$), and amyloid load vs. BACE1 level ($p < 0.05$).

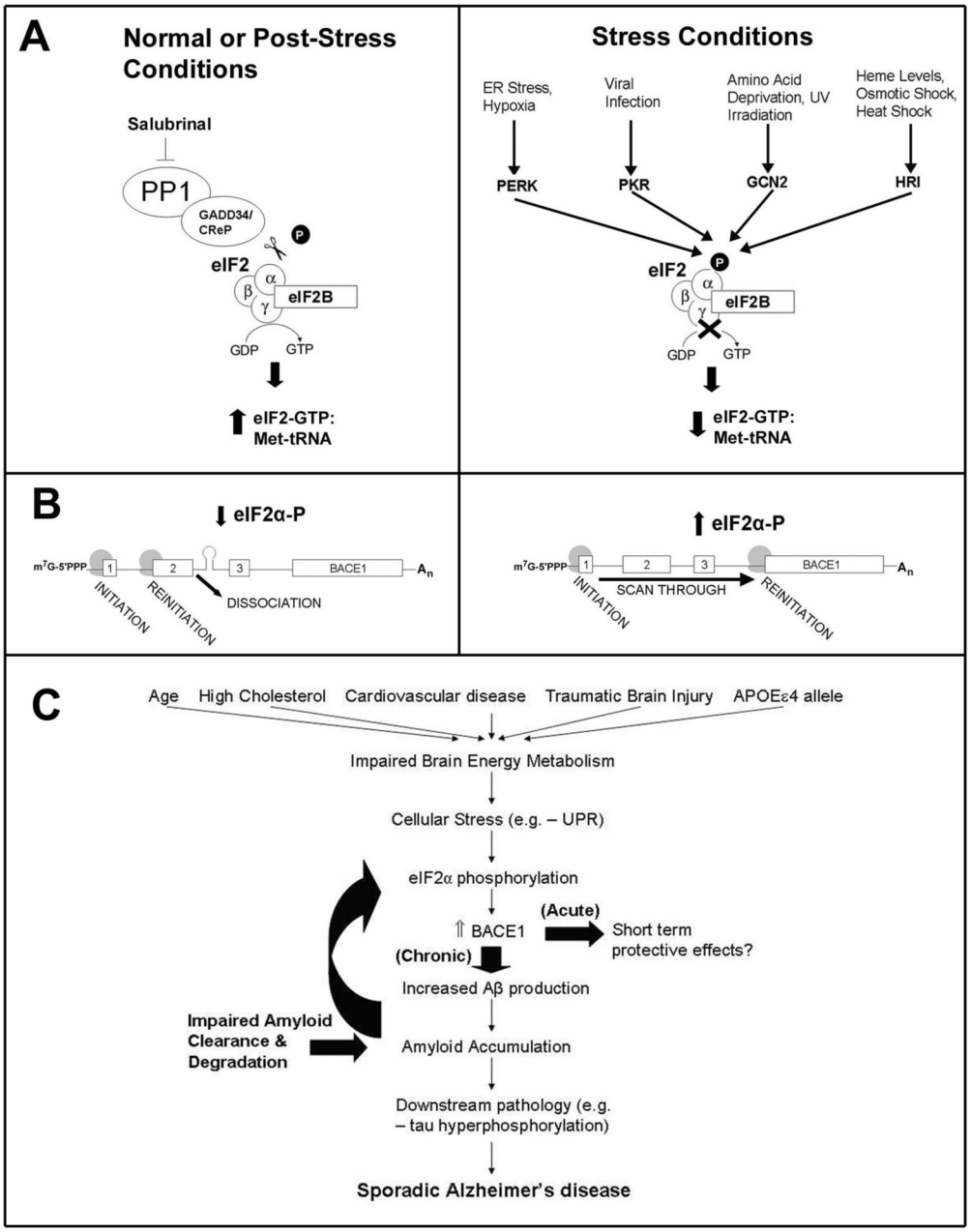

Figure 8. Models for role of eIF2α phosphorylation in regulation of BACE1 mRNA translation and sporadic Alzheimer's disease pathogenesis

(A) eIF2α phosphorylation determines the concentration of ternary complex and consequently the rate of translation initiation. Under normal or post-stress conditions (left), levels of phosphorylated eIF2α are kept at low levels in the cell due to expression of regulatory subunits of the phosphatase PP1 (GADD34 and CReP), which complex with PP1 and direct PP1 phosphatase activity to eIF2α. The drug salubrinal is a selective inhibitor of the PP1 complex and prevents dephosphorylation of eIF2α-P (Boyce et al., 2005). eIF2B, which catalyzes the exchange of GDP for GTP on the γ subunit of eIF2, freely binds to and dissociates from unphosphorylated eIF2α in the eIF2 complex. In its GTP form, eIF2 assembles into a ternary complex along with methionine-charged tRNA (eIF2 GTP Met-tRNAi). The ternary complex

binds to the 40S ribosomal subunit to form the 43S preinitiation complex, which is then capable of initiating translation at an AUG sequence of the appropriate context on an mRNA transcript. Recognition of the start codon catalyzes GTP to GDP on eIF2, which is then released from the 40S subunit for another cycle of translation initiation. When eIF2α-P levels are low, ternary complex concentration is high and normal mRNA transcripts with short 5' UTRs are translated with high efficiency. However, transcripts like BACE1 with long, uORF-containing, secondary structure-rich 5'UTRs are inefficiently translated. Under stress conditions (right), eIF2α is phosphorylated by one of four different kinases in response to various stimuli. In this case, eIF2B does not readily dissociate from phosphorylated eIF2α in the eIF2α complex, inhibiting the exchange of GDP for GTP on the γ-subunit of eIF2 and effectively reducing the concentration of ternary complex. In this case, the translation of normal mRNA transcripts is inhibited, while transcripts with long, uORF-containing, structured 5'UTRs are paradoxically translated with increased efficiency (de-repression). **(B)** Model for eIF2α phosphorylation-dependent de-repression of BACE1 mRNA translation, based on yeast GCN4 mRNA translational regulation (Schroder and Kaufman, 2006). The BACE1 5' UTR is 453 nucleotides long, has three uORFs (numbered boxes), and has predicted stable secondary structure with a free energy of -215.3 kcal/mol (Lammich et al., 2004). uORF2 has been shown to be the major uORF that inhibits BACE1 mRNA translation under normal conditions (Mihailovich et al., 2007; Zhou and Song, 2006). A stable stem-loop structure (indicated by the hair-pin) is predicted downstream of uORF 2 (Fig. S5B). The AUGs of the three ORFs are in favorable contexts, and reports indicate that they are translated (De Pietri Tonelli et al., 2004; Mihailovich et al., 2007; Zhou and Song, 2006). In our model, ribosomes would bind to the BACE1 mRNA cap, scan down the 5' UTR, and initiate translation first at uORF1. After translation of uORF1 is terminated and the 60S subunits dissociate, a proportion of 40S subunits remain attached and continue to scan along the BACE1 mRNA 5' UTR. Under normal conditions, levels of phosphorylated eIF2α are low (left) and ternary complex concentrations are high. In this case, 40S subunits are efficiently reloaded with ternary complexes, 43S preinitiation complexes are rapidly formed, and translation reinitiation occurs at uORF2. A rare proline codon at the 3' end of uORF2 (boxed P; Fig. S5A) and downstream stable secondary structure (Fig. S5B) increase dissociation of ribosomes from the BACE1 mRNA, reducing the proportion of 40S subunits that scan through the BACE1 mRNA 5' UTR and reach the authentic BACE1 start codon for reinitiation. As a consequence, translation of the BACE1 ORF is inefficient under normal conditions. In contrast, under cellular stress conditions, phosphorylated eIF2α levels are high (right) and ternary complex concentrations are low. Translation initiation is still predicted to occur first at uORF1. Following termination of uORF1 translation, a proportion of 40S subunits remain attached and continue scanning the 5' UTR, as in low eIF2α-P conditions. However, when eIF2α-P is high, decreased ternary complex availability causes the 40S subunit to spend more time scanning before becoming reloaded to form the 43S preinitiation complex. As a result, a higher proportion of 40S subunits scan through uORF2-3 and reach the authentic BACE1 AUG to reinitiate translation at the BACE1 ORF. Thus, BACE1 mRNA translation is more efficient under stress conditions than normal. **(C)** Common risk factors for Alzheimer's disease such as age, high cholesterol, cardiovascular disease, traumatic brain injury, and ApoE4 genotype may lead to a state of impaired energy metabolism in the brain. Reduced energy availability activates the eIF2α-P stress-response pathway in neurons. Phosphorylation of eIF2α augments the translation of specific stress response proteins such as BACE1. Increased production of these stress-response proteins presumably enhances the ability of neurons to survive under low-energy conditions. However, if the stress persists, eIF2α phosphorylation and BACE1 levels remain chronically elevated, leading to increased Aβ production. Even a small increase in Aβ generation may have a significant impact on amyloid accumulation over the many years apparently required for AD development. Elevated Aβ levels may, in turn, cause neuronal dysfunction and stress, feeding back and further activating the eIF2α-P stress-response pathway. Over time, and in combination with other exacerbating factors such as impaired Aβ clearance/degradation, Aβ begins to accumulate in

the brain and plaques form. Aβ accumulation may trigger downstream pathology, (e.g., tau hyperphosphorylation), ultimately leading to neurodegeneration associated with sporadic Alzheimer's disease. (UPR = unfolded protein response).