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Article

TNF-α Mediates PKR-Dependent Memory Impairment and Brain IRS-1 Inhibition Induced by Alzheimer's β-Amyloid Oligomers in Mice and Monkeys

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SUMMARY

Alzheimer's disease (AD) and type 2 diabetes appear to share similar pathogenic mechanisms. dsRNA-dependent protein kinase (PKR) underlies peripheral insulin resistance in metabolic disorders. PKR phosphorylates eukaryotic translation initiation factor 2α (eIF2α-P), and AD brains exhibit elevated phospho-PKR and eIF2α-P levels. Whether and how PKR and eIF2α-P participate in defective brain insulin signaling and cognitive impairment in AD are unknown. We report that β-amyloid oligomers, AD-associated toxins, activate PKR in a tumor necrosis factor α (TNF-α)-dependent manner, resulting in eIF2α-P, neuronal insulin receptor substrate (IRS-1) inhibition, synapse loss, and memory impairment. Brain phospho-PKR and eIF2α-P were elevated in AD animal models, including monkeys given intracerebroventricular oligomer infusions. Oligomers failed to trigger eIF2α-P and cognitive impairment in PKR$^{-/-}$ and TNFR1$^{-/-}$ mice. Bolstering insulin signaling rescued phospho-PKR and eIF2α-P. Results reveal pathogenic mechanisms shared by AD and diabetes and establish that proinflammatory signaling mediates oligomer-induced IRS-1 inhibition and PKR-dependent synapse and memory loss.

INTRODUCTION

Recent evidence suggests that Alzheimer's disease (AD) is a novel, brain-specific form of diabetes (de la Monte and Wands, 2008; De Felice, 2013). AD brains exhibit defective insulin signaling with altered levels and cellular distribution of insulin receptors (Moloney et al., 2010). Insulin signaling is central to neuronal survival, regulation of synapse number, and dendritic plasticity (van der Heide et al., 2005; Chiu et al., 2008; McNay and Recknagel, 2011), raising the possibility that deficient insulin signaling may be linked to neuronal dysfunction in AD.

β-amyloid oligomers (AβOs), toxins that accumulate in AD brains and instigate synapse damage (Ferreira and Klein, 2011), remove insulin receptors from the neuronal surface (Zhao et al., 2008; De Felice et al., 2009) and activate c-Jun N-terminal kinase (JNK) to trigger insulin receptor substrate (IRS-1) inhibition (Bomfim et al., 2012). These findings provided initial clues on how impaired neuronal insulin signaling develops in AD (De Felice, 2013). Landmark studies from the diabetes field have established that activation of proinflammatory tumor necrosis factor alpha and JNK signaling (TNF-α/JNK signaling) is a key mechanism leading to peripheral insulin resistance (Hotamisligil et al., 1994, 1996; Gregor and Hotamisligil, 2011). Therefore, it is likely that a molecular parallel exists between defective brain insulin signaling in AD and peripheral insulin resistance in diabetes (Bomfim et al., 2012; De Felice, 2013).

The double-stranded RNA-dependent protein kinase (PKR) is a critical player in the integration of an inflammatory response that leads to peripheral insulin resistance in metabolic disorders,

including diabetes (Ozcan et al., 2004; Hotamisligil, 2010; Nakamura et al., 2010). Elevated levels of phosphorylated PKR and of its key target, eukaryotic translation initiation factor 2α (eIF2α), have been reported in AD brains and in animal models of AD (Hoozemans et al., 2009; Yoon et al., 2012). eIF2α phosphorylation is critical for memory regulation (Costa-Mattioli et al., 2007) and has been recently shown to mediate prion-related neurodegeneration in the hippocampus (Moreno et al., 2012), a memory center that is affected early in AD.

Given the pathophysiological roles of TNF-α and PKR in peripheral insulin resistance (Nakamura et al., 2010; Gregor and Hotamisligil, 2011), we investigated here whether similar proinflammatory mechanisms might underlie neuronal dysfunction in AD. We hypothesized that the TNF-α pathway might cause phosphorylation of PKR and eIF2α-P, IRS-1 inhibition, and impact synapses and memory in AD. We show that phospho-PKR and eIF2α-P are elevated in the brains of a transgenic mouse model of AD, of mice and cynomolgus monkeys given intracerebroventricular (i.c.v.) infusions of AβOs, and in cultured hippocampal neurons exposed to oligomers. AβOs failed to trigger eIF2α-P and cognitive impairment in both $PKR^{-/-}$ and $TNFR1^{-/-}$ mice, as well as in mice treated with infliximab, a TNF-α neutralizing antibody. Salubrinal and thapsigargin, agents that increase eIF2α-P levels and endoplasmic reticulum (ER) stress, respectively, induced memory impairment in mice. Insulin treatment prevented oligomer-induced phosphorylation of PKR and eIF2α-P in hippocampal cultures. Glucagon-like peptide 1 (GLP-1) receptor agonists blocked eIF2α-P in hippocampal cultures and in the brains of transgenic mice and oligomer-injected monkeys. Collectively, results provide the grounds for targeting TNF-α/PKR/eIF2α-P signaling as a potential disease-modifying therapy for AD.

RESULTS

Aβ Oligomers Instigate Neuronal eIF2α-P and Other ER Stress Markers In Vitro and In Vivo

We first explored whether AβOs abnormally activate the unfolded protein response (UPR), which has been described to intersect with inflammatory and stress signaling pathways that lead to peripheral insulin resistance in chronic metabolic diseases (Ozcan et al., 2004, 2006; Hotamisligil, 2010). To this end, we searched for ER stress markers in cell cultures and in different animal models of AD. Using mature cultured hippocampal neurons, we found increased IRE1α-pSer724 in dendrites and cell bodies after exposure of neurons to AβOs for 3 hr (Figures 1A and 1B and S1A available online). Consistent with increased endonuclease activity of IRE1α upon phosphorylation, levels of spliced X box binding protein 1 (XBP1), a downstream effector of IRE1α recently proposed as a connection between UPR and insulin signaling (Park et al., 2010; Winnay et al., 2010), were increased in oligomer-exposed neurons (Figure S1B). Further, AβOs increased dendritic and cell body levels of eIF2α-pSer51 (eIF2α-P) in hippocampal neurons (Figures 1C, 1D, and S1C).

We also detected increased neuronal levels of 78 kDa glucose-regulated protein (GRP78) (also known as binding immunoglobulin protein, Bip), an ER chaperone that is part of the stress response program and is upregulated in AD brains (Hoozemans et al., 2005), in oligomer-exposed neurons (Figure S1D). Prolonged ER stress is known to trigger apoptosis mediated by C/EBP homologous protein (CHOP; also known as growth arrest- and DNA damage-inducible gene 153, GADD153) (Lai et al., 2007). In line with the results described above, CHOP mRNA levels were increased in neurons exposed to AβOs for 24 hr (Figure S1E).

Next, we looked for eIF2α-P in the brains of APPSwe,PS1ΔE9 (APP/PS1) mice. These mice harbor transgenes for human amyloid precursor protein (APP) bearing the Swedish mutation and a deletion mutant form of presenilin 1 (PS1) and present increased Aβ production and cognitive deficits (Jankowsky et al., 2001). APP/PS1 mice displayed increased hippocampal levels of eIF2α-P compared to wild-type animals (Figure 1E).

AβO-Induced PKR Activation Leads to eIF2α Phosphorylation

Four kinases (PKR-like endoplasmic reticulum kinase [PERK], double-stranded RNA-dependent protein kinase [PKR], general control nonrepressed kinase 2 [GCN2], and heme-regulated inhibitor [HRI]) have been reported to phosphorylate eIF2α under stress conditions (Gkogkas et al., 2010). PERK and PKR are the main eIF2α kinases in response to ER stress and inflammation, respectively (Raven and Koromilas, 2008). We therefore examined the roles of both kinases in oligomer-induced eIF2α-P in neurons. In hippocampal neuronal cultures, the distribution of activated PERK (PERK-pThr981) was mainly restricted to cell bodies, and its levels were not altered by exposure to AβOs for 3 hr (Figures S2A and S2B). In contrast, PKR was activated by exposure to AβOs in neuronal cultures (Figures 1F and 1G). AβOs are known to selectively target a subset of neurons in hippocampal cultures (Lacor et al., 2007). Importantly, elevated phospho-PKR levels were found independent of whether or not neurons exhibited oligomers bound to their dendrites (Figure 1H). This indicates that PKR phosphorylation is not triggered by direct binding of oligomers to individual neurons, but rather is instigated by soluble factors released to the medium upon exposure of cultures to AβOs.

To establish the in vivo relevance of these findings, we analyzed levels of active PKR and PERK in the brains of APP/PS1 transgenic mice. Increased phosphorylation of PKR, but not of PERK, was detected in hippocampi of APP/PS1 mice compared to wild-type controls (Figures 1I and S2C). Consistent with the role of PKR in AβO-dependent eIF2α-P, pharmacological inhibition of PKR completely blocked oligomer-induced eIF2α-P as well as IRE1α-pSer724 (Figures 1J and 1K) in cultured hippocampal neurons. We further extended our investigation to the brains of monkeys that received i.c.v. infusions of AβOs. In monkeys, AβOs promoted eIF2α-P (Figures 1L, 1M, S1F, and S1G) and PKR activation in the hippocampus and entorhinal cortex (Figures 1N, 1O, S1H, and S1I).

TNF-α Receptor Activation Lies Upstream of PKR Dysregulation

In metabolic disorders, ER stress has been linked to insulin resistance and proinflammatory TNF-α signaling (Ozcan et al., 2006; Steinberg et al., 2006). To determine whether TNF-α activation was involved in AβO-induced phospho-PKR and eIF2α-P in hippocampal neurons, we first treated cultures with infliximab, a

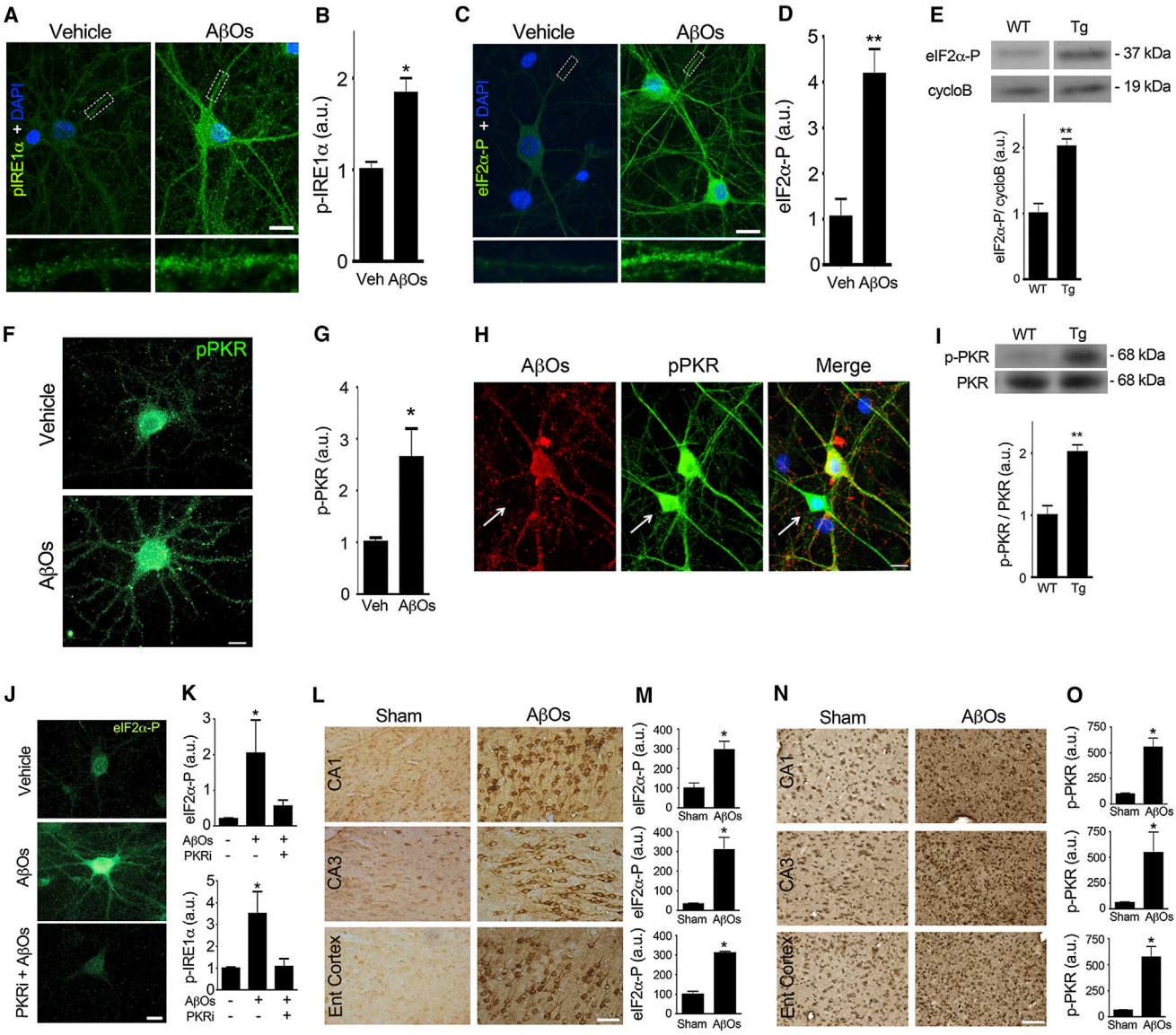

Figure 1. β-Amyloid Oligomers Trigger Neuronal IRE1α-pSer724, eIF2α-P, and Phospho-PKR In Vitro and In Vivo

(A and B) Shown is IRE1α-pSer724 immunolabeling (A) and levels (B) in cultured hippocampal neurons exposed to vehicle or 500 nM AβOs for 3 hr (scale bars = 10 μm). Boxes under each panel show optical zoom images of selected dendrite segments (white dashed rectangles).

(C and D) Shown is eIF2α-P immunolabeling (C) and levels (D), determined from 4–6 experiments using independent cultures (30 images analyzed per experimental condition per experiment).

(E) Immunoblot analysis of eIF2α-P in hippocampal homogenates from 13- to 16-month-old WT (n = 5) or APP/PS1 (n = 7) mice.

(F) Immunolabeling for PKR-pThr451 in cultured hippocampal neurons exposed to vehicle or 500 nM AβOs for 3 hr (scale bar = 10 μm).

(G) PKR-pThr451 immunofluorescence levels, determined from four experiments using independent cultures (30 images analyzed per experimental condition per experiment).

(H) Immunolabeling of DAPI (blue), AβOs (red), and phospho-PKR (green) in cultured hippocampal neurons. White arrow indicates a neuron with elevated phospho-PKR level in the absence of oligomer binding.

(I) Immunoblot analysis of PKR-pThr451 (normalized by total PKR) in hippocampal homogenates of 13- to 16-month-old WT (n = 5) and APP/PS1 (n = 7) mice. *$p < 0.05$, **$p < 0.01$, Student's t test.

(J) eIF2α-P immunolabeling in hippocampal neurons exposed to vehicle, 500 nM AβOs, or 1 μM PKR inhibitor + 500 nM AβOs for 3 hr (scale bar = 10 μm).

(K) eIF2α-P and IRE1α-pSer724 immunofluorescence levels, determined from three experiments using independent cultures. *$p < 0.05$, ANOVA followed by Bonferroni post hoc test.

(L and M) Shown is eIF2α-P immunolabeling (L) and integrated optical densities (M) in hippocampus (CA1 and CA3 regions) and entorhinal cortex of cynomolgus monkeys that received i.c.v. injections of AβOs (n = 4) (right panels) compared to sham-operated control monkeys (n = 3) (left panels) (scale bars = 200 μm).

(N and O) Shown is phosphor-PKR labeling (N) and integrated optical densities (O) (see Experimental Procedures) in hippocampus (CA1 and CA3 regions) and entorhinal cortex of sham-operated (Sham) (n = 3) or AβO-injected monkeys (AβOs) (n = 4). *$p < 0.05$, ANOVA followed by Bonferroni post hoc test. Graphs show means ± SEM. See also Figure S1.

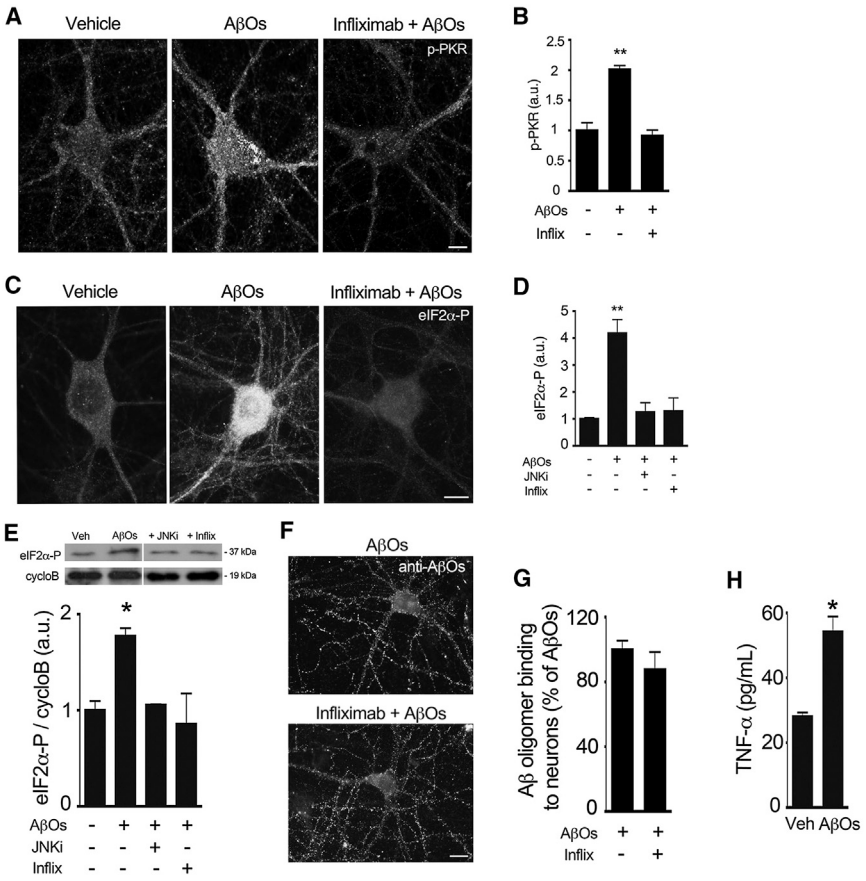

Figure 2. TNF-α Mediates Oligomer-Induced Phospho-PKR and eIF2α-P

(A) PKR-pThr451 immunolabeling in cultured hippocampal neurons exposed for 3 hr to vehicle or 500 nM AβOs in the absence or presence of infliximab.

(B) PKR-pThr451 immunofluorescence levels, determined from four experiments using independent cultures.

(C) eIF2α-P immunolabeling in cultured hippocampal neurons exposed for 3 hr to vehicle or 500 nM AβOs in the absence or presence of infliximab.

(D) eIF2α-P immunofluorescence levels, determined from four experiments using independent cultures exposed to vehicle or 500 nM AβOs in the absence or presence of infliximab or JNK inhibitor (JNKi). **$p < 0.01$, ANOVA followed by Bonferroni post hoc test.

(E) Immunoblot analysis for eIF2α-P in hippocampal neuronal cultures exposed for 3 hr to 500 nM AβOs in the absence or in the presence of infliximab or JNK inhibitor ($n = 3$ independent experiments; *$p < 0.05$, Student's t test).

(F) Imunolabeling of AβOs (with anti-AβO, NU4) in neurons exposed to 500 nM AβOs in the absence or presence of infliximab.

(G) Quantification of Aβ oligomer binding in neurons exposed to AβOs in the absence or presence of infliximab.

(H) Soluble TNF-α levels in conditioned media from cultured hippocampal neurons exposed to vehicle (veh) or 500 nM AβOs for 3 hr ($n = 3$ independent experiments; *$p < 0.05$, Student's t test). Graphs show means ± SEM. See also Figure S2.

TNF-α neutralizing monoclonal antibody. Infliximab suppressed both PKR activation and eIF2α-P triggered by oligomers (Figures 2A–2E). SP600125, a specific JNK inhibitor, also blocked eIF2α-P in neuronal cultures (Figures 2D and 2E), in line with recent studies that have implicated the JNK pathway in AβO-induced IRS-1 inhibition (Ma et al., 2009; Bomfim et al., 2012; De Felice, 2013). It is notable that infliximab did not block oligomer binding to neurons (Figures 2F and 2G), substantiating the notion that activation of TNF-α/PKR signaling is independent of direct binding of AβOs to individual neurons and is likely mediated by TNF-α secreted to the medium. Indeed, TNF-α levels were increased in the culture medium after exposure to oligomers (Figure 2H), consistent with our recent finding that TNF-α levels are increased in the brains of mice that received i.c.v infusions of oligomers (Ledo et al., 2013).

Activation of the TNF-α/PKR/eIF2α-P Pathway Is Linked to Synapse Loss and Memory Impairment

We next hypothesized that activation of proinflammatory TNF-α signaling might be connected to AβO-induced memory impairment. Supporting this hypothesis, infliximab prevented memory impairment triggered by AβOs in mice (Figure 3A). We next injected oligomers i.c.v. in TNFR1$^{-/-}$ mice. Significantly, AβOs caused memory deficits in wild-type (WT) mice, but not in TNFR1$^{-/-}$ mice (Figures 3B, 3C, and S3). These results implicate TNF-α signaling in the mechanism underlying memory impairment induced by AβOs in mice. Interestingly, AβOs triggered phosphorylation of PKR and eIF2α in the hippocampus of WT mice, but not in TNFR1$^{-/-}$ mice (Figures 3D and 3E), establishing that activation of TNF-α receptors lies upstream of PKR and eIF2α-P in vivo.

Synapse loss has been proposed to be the best pathological correlate of the extent of dementia in AD (Terry et al., 1991), and AD brains present reduced levels of synaptophysin and PSD-95, pre- and postsynaptic markers, respectively (Terry et al., 1991). AβOs have been shown to reduce the levels of synaptophysin or PSD-95 in neuronal cultures, in the brains of mice receiving i.c.v. infusions of oligomers, and in ex vivo human cortical slices (Sebollela et al., 2012; Figueiredo et al., 2013). Supporting the notion that synaptic deterioration underlies TNF-α-dependent memory impairment induced by AβOs, levels of synaptophysin and PSD-95 were decreased in hippocampi of WT, but not of TNFR1$^{-/-}$, mice i.c.v. injected with AβOs (Figure 3F).

To determine the role of PKR in AβO-induced cognitive impairment, we investigated the effect of AβOs in PKR$^{-/-}$ mice. Importantly, we found that AβOs induced hippocampal eIF2α-P and cognitive deficits in WT mice, but not in PKR$^{-/-}$ mice (Figures 3G–3I). These results demonstrate that PKR, recently implicated in metabolic stress and impaired insulin signaling in diabetes (Nakamura et al., 2010), is a key mediator of neuronal eIF2α-P and memory impairment induced by AβOs.

To further examine the impact of PKR activation on synapses, we exposed hippocampal cultures to AβOs in the absence or presence of a PKR inhibitor. After 24 hr, we evaluated synapse

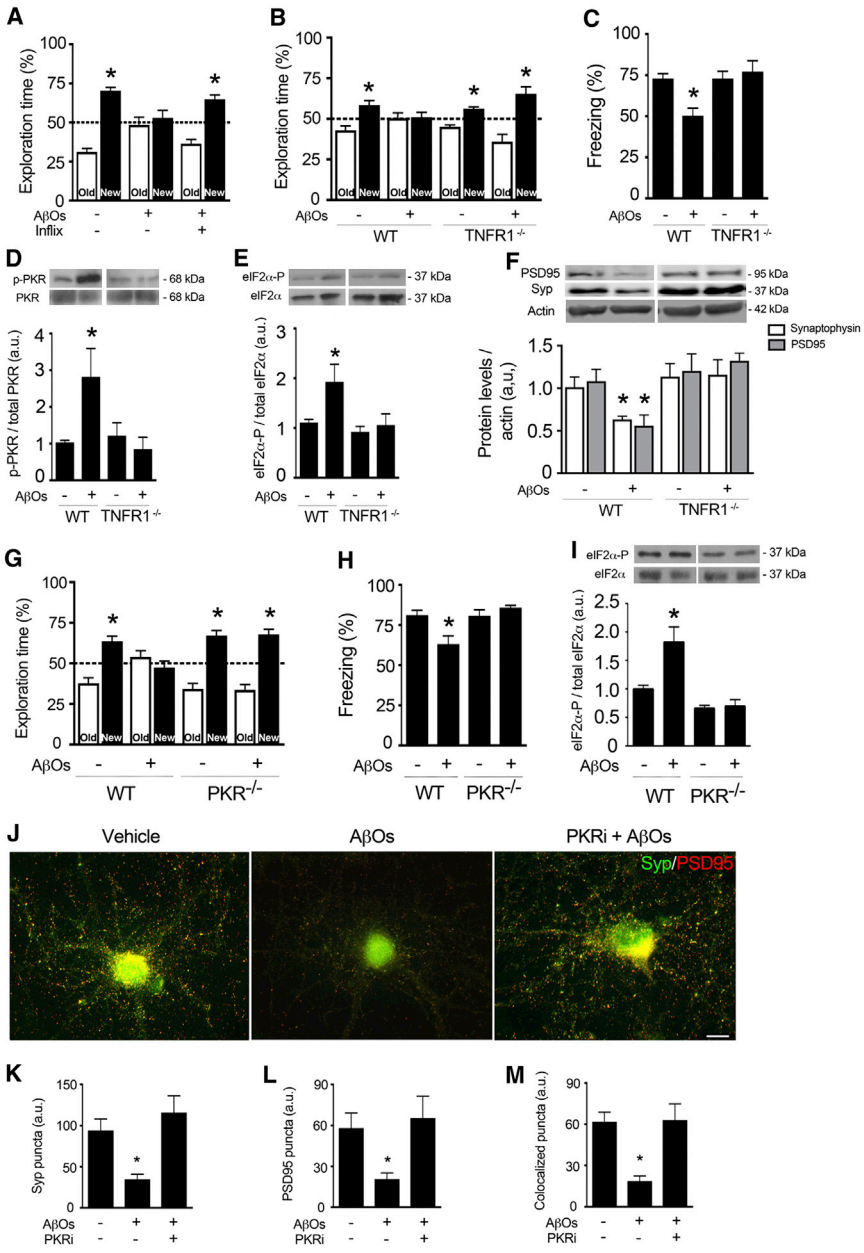

Figure 3. TNF-α/PKR Signaling Mediates Cognitive Impairment and Synapse Loss Caused by Aβ Oligomers

(A) Exploration times of mice i.c.v. injected with vehicle or 10 pmol AβOs in the absence or presence of infliximab in the novel object recognition task (n = 7–10 per experimental group).

(B) Exploration times of WT or TNFR1$^{-/-}$ mice i.c.v. injected with vehicle or 10 pmol AβOs in the novel object recognition task (n = 9–10 per experimental group). Asterisks denote a statistically significant ($p < 0.05$) difference from 50% (reference value).

(C) Freezing times of WT or TNFR1$^{-/-}$ mice injected with vehicle or 10 pmol AβOs in the contextual fear conditioning task. n = 9–10 animals per experimental group. *$p < 0.05$, ANOVA followed by Bonferroni post hoc test.

(D–F) Immunoblot analysis of PKR-pThr451 (normalized by total PKR) (D), eIF2α-P (normalized by total eIF2α) (E), and synaptophysin (white bars) and PSD95 (gray bars) (normalized by β-actin) (F) in hippocampal homogenates of 2- to 3-month-old WT or TNFR1$^{-/-}$ mice injected with vehicle or 10 pmol AβOs (n = 7 for each experimental condition).

(G) Exploration times of WT or PKR$^{-/-}$ mice exposed to vehicle or 10 pmol AβOs in the novel object recognition task (n = 8–10 per experimental group). Asterisks denote a statistically significant difference ($p < 0.05$) from 50% (reference value).

(H) Freezing times of WT or PKR$^{-/-}$ mice injected with vehicle or 10 pmol AβOs in the contextual fear conditioning task. n = 9–10 animals per experimental group. *$p < 0.05$, ANOVA followed by Bonferroni post hoc test.

(I) Immunoblot analysis of eIF2α-P (normalized by total eIF2α) in hippocampal homogenates of 2- to 3-month-old WT or PKR$^{-/-}$ mice injected with vehicle or 10 pmol AβOs (n = 7 for each experimental condition).

(J) Synaptophysin (green) and PSD95 (red) immunolabeling of cultured hippocampal neurons exposed for 3 hr to vehicle or 500 nM AβOs in the absence or presence of PKR inhibitor. Synapses, evidenced by colocalized puncta, appear in yellow.

(K–M) Quantification of synaptophysin (K), PSD95 (L), and colocalized (M) puncta, determined from four experiments using independent cultures. *$p < 0.05$, ANOVA followed by Bonferroni post hoc test. Graphs show means ± SEM. See also Figure S3.

density by determining immunoreactivities of synaptophysin and PSD-95 and their colocalization at synapses. Results showed that inhibition of PKR attenuated synapse loss induced by AβOs (Figures 3J–3M) and suggest that loss of synaptic proteins underlies the deleterious effects of PKR on memory.

Based on the findings described above, we hypothesized that elevated eIF2α-P in the brain might be linked to memory impairment triggered by AβOs. To explore the connection between eIF2α-P and memory, we performed i.p. injections of salubrinal (an inhibitor of GADD34, a phosphatase that preferentially dephosphorylates eIF2α) to increase eIF2α-P levels in the hippocampi of adult Swiss mice. Animals that received salubrinal presented memory impairment in the novel object recognition task (Figure 4A) and significantly lower hippocampal levels of PSD-95 and synaptophysin (Figures 4B–4D). We also found decreased levels of synaptophysin and PSD-95, as well as decreased synapse density, in salubrinal-exposed hippocampal cultures (Figures 4E–4H). Control experiments indicated that, as expected, salubrinal promoted eIF2α-P in hippocampal neurons in culture (Figures S4A and S4B) and in the brains of injected mice (Figure S4C). These results show that eIF2α-P is sufficient to cause memory impairment and synapse loss in mice.

Additional support to the notion that eIF2α-P and, more generally, ER stress cause memory impairment came from the observation that thapsigargin, a classical inducer of ER stress that triggers eIF2α-P in neurons (Figures S5A and S5B), caused brain eIF2α-P and memory impairment when injected i.c.v. in mice (Figures S5C and S5D). 4-phenylbutyrate (4-PBA), a chemical

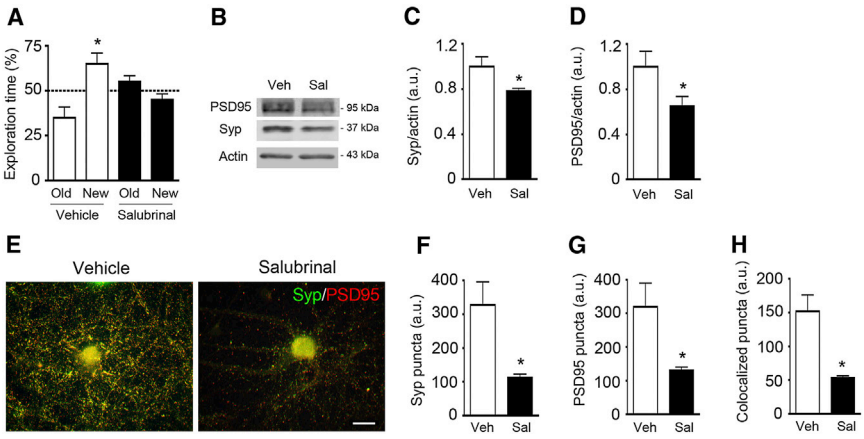

Figure 4. eIF2α-P Triggers Cognitive Impairment and Synapse Loss

(A) Exploration times of mice i.p. injected for 7 days with vehicle or 1 mg/kg salubrinal in the novel object recognition task (n = 10 per experimental group). Asterisks denote a statistically significant difference ($p < 0.05$) from 50% (reference value).

(B) Representative immunoblot analysis of synaptophysin and PSD-95 levels in hippocampal homogenates of 2- to 3-month-old mice receiving vehicle or salubrinal intraperitoneally for 7 days (n = 7 per experimental group).

(C and D) Levels of synaptophysin (C) and PSD-95 (D) were normalized by β-actin.

(E) Synaptophysin (green) and PSD95 (red) immunolabeling in cultured hippocampal neurons exposed for 3 hr to vehicle or 10 μM salubrinal. Synapses, evidenced by colocalized puncta, appear in yellow.

(F–H) Quantification of synaptophysin (F), PSD-95 (G), and colocalized (H) puncta, determined from four experiments using independent cultures. *$p < 0.05$, Student's t test. Graphs show means ± SEM. See also Figure S4.

chaperone known to alleviate ER stress, prevented memory loss (Figure S5D). 4-PBA further blocked memory impairment caused by i.c.v. injection of AβOs in mice (Figure S5E), implicating ER stress in the impact of AβOs on memory.

TNF-α, but Not eIF2α-P, Causes Neuronal IRS-1 Inhibition

We recently reported that TNF-α and PKR mediate IRS-1 inhibition in cultured hippocampal neurons (Bomfim et al., 2012). Establishing that proinflammatory TNF-α mediates oligomer-induced IRS-1 inhibition in vivo, AβOs triggered IRS-1pSer636 in the hippocampus of WT mice, but not in TNFR1$^{-/-}$ mice (Figure 5A). We also examined IRS-1pSer levels in the brains of PKR$^{-/-}$ mice. Possibly reflecting different patterns of activation of IRS-1 in mice of different genetic backgrounds (Xu et al., 2013), we did not detect increased IRS-1pSer levels induced by AβOs in WT 129/SvEv mice. Interestingly, however, lower IRS-1pSer levels were found in PKR$^{-/-}$ mice than in WT mice, demonstrating that suppression of PKR per se attenuates IRS-1 inhibition (Figure 5B).

Because ER stress and eIF2α-P are linked to insulin resistance in peripheral tissues (Ozcan et al., 2004; Birkenfeld et al., 2011), we next aimed to determine whether they might be related to IRS-1 inhibition in neurons. We initially found that thapsgargin triggered dendritic IRS-1pSer636 in hippocampal neurons, and this was blocked by 4-PBA (Figures 5C and 5D). However, levels of IRS-1pSer were unaffected in hippocampal neuronal cultures exposed to salubrinal (Figures 5E and 5F) or in hippocampi of salubrinal-injected mice (Figures 5G and 5H). These results suggest that, while eIF2α-P alone is not sufficient to cause IRS-1pSer in neurons, aberrant activation of TNF-α signaling and induction of ER stress play important roles in brain IRS-1 inhibition. In this regard, it is interesting to note that AβOs induced upregulation of XBP1s, GRP78, and CHOP (Figures S1B, S1D, and S1E), implicated in mechanisms by which ER dysfunction is linked to inflammatory signaling and insulin resistance (Hotamisligil, 2010). Based on our results, it is conceivable that different branches of the UPR respond to AβO-induced TNFR activation and lead to IRS-1 inhibition.

Stimulation of Insulin Signaling Prevents Phospho-PKR and eIF2α-P

Lastly, we asked whether antidiabetic agents, recently shown to restore insulin signaling and exert neuroprotective actions in AD models (De Felice et al., 2009; McClean et al., 2011; Bomfim et al., 2012), could alleviate phospho-PKR and eIF2α-P triggered by AβOs. Insulin treatment blocked the phosphorylation of PKR and eIF2α in hippocampal cultures exposed to AβOs (Figures 6A–6D). Exendin-4, a GLP-1 receptor agonist approved for treatment of diabetes (Ryan and Hardy, 2011), blocked AβO-triggered eIF2α-P in hippocampal neurons (Figures 6C and 6D), and intraperitoneal treatment with exendin-4 rescued eIF2α-P levels in hippocampi of APP/PS1 mice (Figure 6E).

Liraglutide, a long-lasting GLP-1 receptor agonist, has also been shown to exhibit neuroprotective actions in animal models. Liraglutide treatment significantly reduced the levels of ER stress marker GRP78 in the brains of APP/PS1 mice (Figure S6A). This was accompanied by a significant increase in levels of synaptic marker drebrin (Figure S6B), suggesting increased synaptic density. We further asked whether liraglutide would affect hippocampal Aβ oligomer burden in APP/PS1 mice. Interestingly, levels of 28 kDa and 108 kDa Aβ oligomers, recently implicated in AD pathogenesis (Tomiyama et al., 2010; Bao et al., 2012), were markedly reduced in liraglutide-treated animals (Figures S6C and S6D).

The neuroprotective actions of exendin-4 and liraglutide translated into memory benefit, as indicated by improved performance in fear conditioning learning in APP/PS1 mice (Figures S6E and S6F). These findings are in line with recent studies that demonstrated beneficial effects of GLP-1R activation on memory in AD mice (McClean et al., 2011; Bomfim et al., 2012). Control measurements showed that neither exendin-4 nor liraglutide altered animal body weight during experiments (Figures S6G and S6H). Collectively, these data suggest that

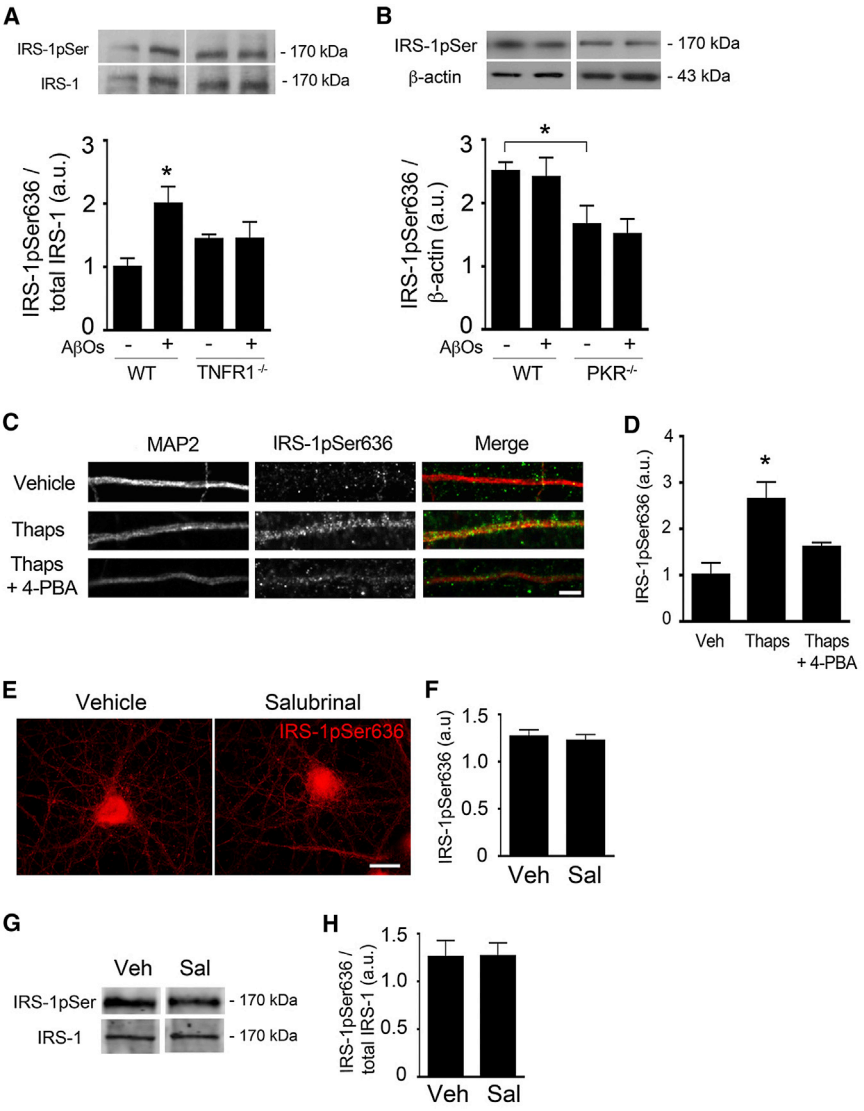

Figure 5. TNF-α and ER Stress, But Not eIF2α-P Alone, Cause Neuronal IRS-1 Inhibition

(A) Immunoblot analysis of IRS-1pSer636 (normalized by total IRS-1) in hippocampal homogenates of 2- to 3-month-old WT or TNFR1$^{-/-}$ mice injected with vehicle or 10 pmol AβOs (n = 7 for each experimental condition).

(B) Immunoblot analysis of IRS-1pSer636 in hippocampal homogenates of 2- to 3-month-old WT or PKR$^{-/-}$ mice injected with vehicle or 10 pmol AβOs (n = 8 for each experimental condition).

(C) MAP2 and IRS-1pSer636 immunolabeling in dendrite segments from hippocampal neurons exposed to vehicle, 1 μM thapsigargin, or 1 mM 4-PBA + 1 μM thapsigargin for 3 hr.

(D) Graph shows IRS-1pSer636 immunofluorescence levels (3 independent experiments; 80 dendrite segments analyzed per experimental condition per experiment). Scale bar: 5 μm.

(E) IRS-1pSer636 immunolabeling in cultured hippocampal neurons exposed to vehicle or 10 μM salubrinal for 3 hr (scale bars = 10 μm).

(F) Graph shows IRS-1pSer636 immunofluorescence levels (3 independent experiments; 30 images/experimental condition/experiment).

(G) Representative immunoblot analysis of IRS-1pSer636 (normalized by total IRS-1) in hippocampal homogenates of 2- to 3-month-old mice injected intraperitoneally with vehicle or 1 mg/kg salubrinal (n = 7 per experimental condition).

(H) IRS-1pSer636 levels were normalized by total IRS-1. *$p < 0.05$, ANOVA followed by Bonferroni post hoc test. Graphs show means ± SEM. See also Figure S5.

the beneficial actions of GLP-1R agonists in AD transgenic mice involve decreased AβO levels and attenuation of brain eIF2α-P and ER stress. Finally, systemic treatment with liraglutide reduced eIF2α-P induced by AβOs in the entorhinal cortex and hippocampus of two cynomolgus monkeys (Figures 6F–6H).

DISCUSSION

An intriguing molecular connection has been established between type 2 diabetes and AD, following the discovery that impaired insulin signaling, a hallmark of diabetes, is present in AD brains (Bomfim et al., 2012; Talbot et al., 2012). Clinical and epidemiological studies have further linked AD and diabetes, with each disease increasing the risk of developing the other (Ott et al., 1996; Janson et al., 2004). AβOs, synaptotoxins that accumulate in AD brains (Gong et al., 2003), were recently found to disrupt neuronal insulin signaling by causing cellular redistribution of insulin receptors and inhibitory serine phosphorylation of IRS-1 (Zhao et al., 2008; De Felice et al., 2009; Bomfim et al., 2012). These studies have provided initial evidence that mechanisms similar to those underlying peripheral insulin resistance in metabolic diseases lead to impaired brain insulin signaling in AD (Bomfim et al., 2012; Talbot et al., 2012; De Felice, 2013).

AβOs exert multiple neurotoxic actions, including disruption of neuronal calcium homeostasis (De Felice et al., 2007; Mattson, 2010), abnormal ER calcium release (Paula-Lima et al., 2011), and activation of JNK (De Felice et al., 2009; De Felice, 2013), conditions known to favor the development of ER stress in peripheral tissues (Hotamisligil, 2010). ER stress plays a key role in metabolic disorders, including type 2 diabetes and obesity, and is linked to peripheral insulin resistance and inflammation (Ozcan et al., 2004). Elevated ER stress markers, including eIF2α-P, have been reported in AD brains (Hoozemans et al., 2009; Yoon et al., 2012). Using different experimental models, including monkeys that received i.c.v. injections of AβOs, we demonstrate here that eIF2α-P and other ER stress responses are induced by AβOs in neurons. Our findings are in accord with studies that reported elevated levels of ER stress markers in other cellular and animal models of AD (Yoon et al., 2012) and indicate that pathological findings in those studies can be attributed to the toxic impact of AβOs.

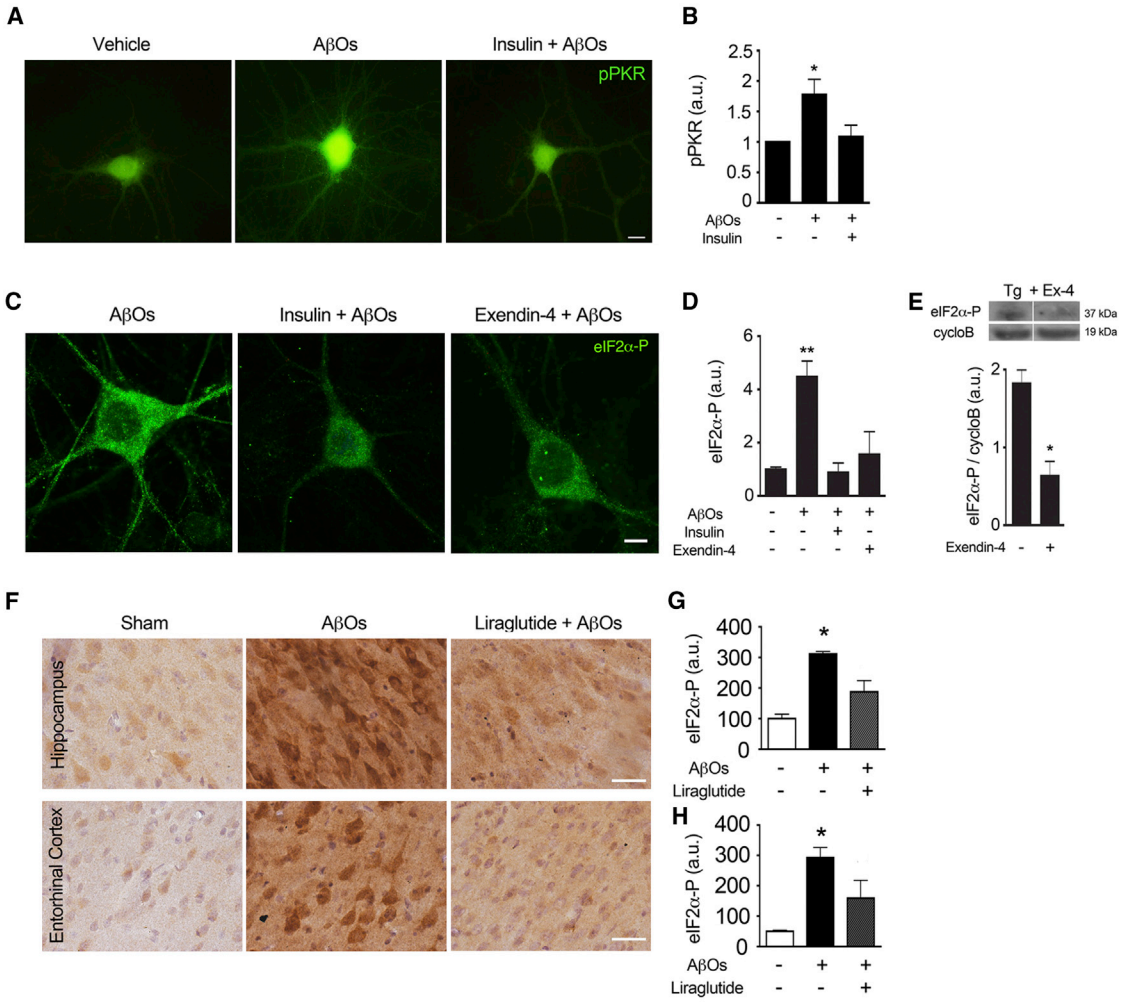

Figure 6. Antidiabetic Agents Block AβO-Induced eIF2α-P In Vitro and In Vivo

(A) PKR-pThr451 immunolabeling in cultured hippocampal neurons exposed for 3 hr to vehicle or 500 nM AβOs in the absence or presence of 1 μM insulin (scale bar = 10 μm).

(B) PKR-pThr451 immunofluorescence levels, determined from four experiments using independent cultures.

(C) eIF2α-P immunolabeling in hippocampal neurons exposed for 3 hr to 500 nM AβOs, 1 μM insulin + 500 nM AβOs, or 300 nM exendin-4 + 500 nM AβOs (scale bar = 10 μm).

(D) eIF2α-P immunofluorescence levels (n = 3 experiments using independent cultures; 30 images analyzed per experimental condition per experiment). **p < 0.01, ANOVA followed by Bonferroni post hoc test.

(E) Immunoblot analysis for eIF2α-P in hippocampal homogenates from 13- to 16-month-old APP/PS1 (n = 7) or exendin-4-treated APP/PS1 (n = 5) mice. Graphs show means ± SEM.

(F) eIF2α-P immunolabeling in the hippocampi (representative images from CA3) and entorhinal cortices of sham, AβO-injected, or liraglutide-treated and AβO-injected monkeys (scale bars = 200 μm).

(G and H) eIF2α-P immunolabeling densities in the hippocampi (G) and entorhinal cortices (H) of sham (white bar), AβO-injected (black bar), or liraglutide-treated AβO-injected monkeys (gray bars). Graphs show means ± SEM obtained for different animals in each experimental group (n = 3, 4, or 2 for sham, AβO-injected, or liraglutide-treated AβO-injected monkeys, respectively). Asterisk indicates a statistically significant ($p < 0.05$; Student's t test) difference between sham and AβO-injected animals. See also Figure S6.

We have identified PKR as an eIF2α kinase activated by AβOs in neurons and shown that AβOs act via the TNF-α pathway to activate PKR. Active PKR, present in AD brains (Bullido et al., 2008), is an important mediator of inflammation and IRS-1 dysfunction in metabolic diseases (Nakamura et al., 2010; Gregor and Hotamisligil, 2011). Of direct relevance to the current study, PKR was recently shown to be involved in neuronal IRS-1 inhibition triggered by AβOs (Bomfim et al., 2012) and to negatively regulate memory in mice (Zhu et al., 2011). Current findings indicate that PKR activation does not depend on direct binding of oligomers to individual neurons, as elevated phospho-PKR and eIF2α-P levels were detected in neurons regardless of whether or not they had oligomers bound to their dendrites. Therefore, AβOs do not seem to act directly on neurons to phosphorylate PKR. Rather, it is likely that a crosstalk between neurons and microglia leads to elevated levels of TNF-α, causing

activation of neuronal TNF-α/PKR/eIF2α signaling. TNF-α has been suggested recently to play a role in brain dysfunction in different diseases, including AD, trauma, and Parkinson's disease (Clark et al., 2012). Elevated TNF-α levels are further thought to cause cognitive deficits (He et al., 2007). Our results demonstrate that, in Alzheimer's disease, elevated TNF-α is a consequence of the brain accumulation and impact of AβOs.

AD is a devastating disease affecting memory. AβOs are recognized as potent synaptotoxins that inhibit synaptic plasticity (Ferreira and Klein, 2011). We found here that oligomer-induced aberrant TNF-α/PKR/eIF2α signaling and induction of ER stress are linked to synapse loss and memory impairment. It is notable that, in both PKR$^{-/-}$ and TNFR1$^{-/-}$ mice, oligomers failed to trigger eIF2α-P and cognitive impairment. Inhibition of PKR was found to attenuate the loss of PSD-95 and synaptophysin induced by AβOs, implicating synapse deterioration in the deleterious impact of PKR on memory. PKR and eIF2α have both been shown to be critical for memory regulation (Costa-Mattioli et al., 2007; Zhu et al., 2011). More recently, eIF2α-P was found to mediate prion-related neurodegeneration in the hippocampus (Moreno et al., 2012). We report that upregulation of eIF2α-P triggers synapse loss, suggesting that synergistic neurotoxic events that culminate in eIF2α-P may respond, at least in part, for synapse damage in AD. Our results thus establish that AβO-induced TNFα, PKR, and eIF2α-P dysregulation is directly linked to synapse failure and cognitive impairment, revealing a mechanism by which AβOs disrupt memory in AD.

The parallel we now describe between AD and diabetes sheds light on how insulin signaling is impaired in AD. In peripheral tissues, inflammatory and metabolic stress signaling cascades trigger disruption of insulin signaling (Ozcan et al., 2004, 2006; Hotamisligil, 2010; Nakamura et al., 2010; Gregor and Hotamisligil, 2011). Our findings show that ER stress is linked to neuronal IRS-1 inhibition and may act synergistically with proinflammatory signals to disrupt brain insulin signaling in AD.

It is important to note that our conclusion that TNF-α signaling is linked to both defective brain insulin signaling and memory impairment caused by AβOs does not necessarily imply that memory impairment is a direct consequence of disruption of insulin signaling. In fact, the role of insulin signaling in memory formation, and how defective signaling might result in neurodegenerative disorders, is still under investigation. While insulin has been shown to positively modulate hippocampal synaptic plasticity (van der Heide et al., 2005), an early study showed that spatial memory was preserved in neural-specific insulin receptor β subunit knockout (NIRKO) mice (Schubert et al., 2004). A recent study, however, reported that IRβ haploinsufficiency causes reduced brain insulin signaling and defects in late-phase long-term potentiation (LTP) and long-term recognition memory storage, thereby implicating insulin receptor (IR)-dependent mechanisms in memory formation (Nisticò et al., 2012). Significantly, intranasal insulin administration has been found to enhance memory in healthy volunteers (Benedict et al., 2004), in memory-impaired older subjects (Reger et al., 2006), and in early AD patients (Craft et al., 2012), with beneficial effects reported for both acute and long-term insulin treatment.

In sporadic AD (which corresponds to >90% of AD cases), the exact mechanism that leads to accumulation of Aβ oligomers and amyloid in the brain remains to be fully elucidated. Interestingly, eIF2α-P promotes BACE1 expression, Aβ production, and deposition (O'Connor et al., 2008). Thus, AβO-induced TNF-α, PKR, and eIF2α-P may constitute a hub in a feedforward deleterious cycle involving increased AβO generation and perpetuation and amplification of neuronal dysfunction.

In line with our current findings demonstrating that eIF2α-P is sufficient to cause synapse loss and memory impairment in mice, a very recent study showed that the eIF2α kinase PERK mediates Aβ-induced LTP impairment (Ma et al., 2013). Importantly, Ma and colleagues further reported that genetically suppressing the eIF2α kinases PERK or GCN2 prevented spatial memory impairment in AD mice (Ma et al., 2013). Although the complete set of mechanisms remains to be elucidated, it is conceivable that TNF-α represents an initial trigger that orchestrates activation of multiple stress response pathways (of which the PKR/eIF2α pathway here investigated appears central) that culminate with synapse dysfunction and memory loss in AD.

In recent years, there has been a strong effort to develop new therapeutic strategies for diabetes and insulin resistance disorders. GLP-1 receptor stimulation has insulinotropic action and restores glucose homeostasis in peripheral tissue (Yusta et al., 2006), and several GLP-1 analogs are currently used for diabetes treatment. We demonstrate here that both insulin and GLP-1R agonists prevent abnormal neuronal phospho-PKR and eIF2α-P. The observation that insulin protects neurons from AβO-induced PKR activation suggests that blockade of eIF2α-P by insulin is mediated by suppression of PKR. Recent studies have shown beneficial effects of exendin-4 and liraglutide against neurodegeneration and cognitive impairment in AD models (Li et al., 2010; McClean et al., 2011; Bomfim et al., 2012), but the signaling mechanisms involved in neuroprotection are only partially known. The present study suggests a mechanism involving suppression of the PKR/eIF2α-P pathway. Importantly, we provide evidence that a GLP-1R agonist exerts neuroprotective actions in the primate brain, as systemic liraglutide treatment reduced oligomer-induced brain eIF2α-P in monkeys.

In conclusion, our findings establish that activation of TNF-α signaling mediates AβO-induced brain IRS-1 inhibition and PKR-dependent eIF2α-P, synapse loss, and memory impairment (Figure 7), revealing mechanisms that lead to synapse loss and memory impairment. Identifying a pathogenic mechanism that is shared between AD and diabetes and contributes to memory loss in AD may open avenues for rapid implementation of clinically approved antidiabetic drugs as therapeutics in AD.

EXPERIMENTAL PROCEDURES

Aβ Oligomers

Oligomers were prepared weekly from synthetic $A\beta_{1-42}$ and routinely characterized by size-exclusion chromatography and, occasionally, by western immunoblots and transmission electron microscopy, as previously described (De Felice et al., 2007, 2008; Sebollela et al., 2012). Oligomers were kept at 4°C and used within 48 hr of preparation.

Mature Hippocampal Cultures

Primary rat hippocampal neuronal cultures, prepared and developed in Neurobasal Medium supplemented with B27 (Invitrogen) and antibiotics according to established procedures (De Felice et al., 2007), were used after

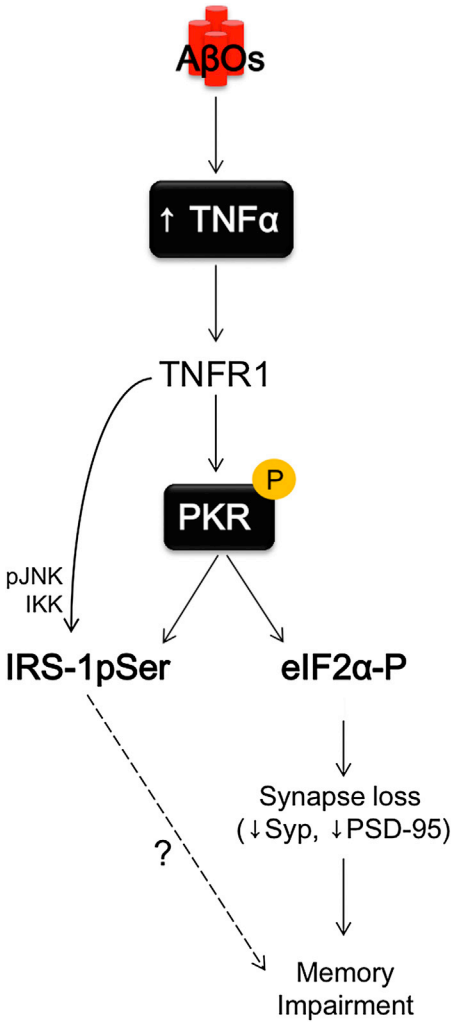

Figure 7. Aβ Oligomers Trigger Synapse Loss, Memory Impairment, and IRS-1 Inhibition via TNF-α/PKR Signaling

Aβ oligomers lead to increased brain levels of TNF-α, leading to TNFR1-mediated activation of PKR and other stress kinases. Activated PKR phosphorylates neuronal IRS-1 (see also Bomfim et al., 2012) and eIF2α. Increased eIF2α-P levels trigger synapse loss and memory impairment.

18–21 days in vitro. All procedures were approved by the Institutional Animal Care and Use Committee of the Federal University of Rio de Janeiro (protocol #IBqM 022). Cultures were exposed at 37°C for 3 hr to 500 nM Aβ oligomers or an equivalent volume of vehicle (2% DMSO in phosphate-buffered saline [PBS]). When present, PKR inhibitor (1 μM), insulin (1 μM), exendin-4 (300 nM), SP600125 (10 μM), or infliximab (1 μg/mL) were added to cultures 30 min before Aβ oligomers. For ER stress induction, thapsigargin (1 μM) was added to neuronal cultures for 3 hr, and in some experiments 4-phenylbutyrate (4-PBA) (1 μM) was also present. For eIF2α-P induction, salubrinal (10 μM) was added to neuronal cultures for 3 hr.

Experimental Subjects

Male Swiss mice were obtained from our animal facility (Federal University of Rio de Janeiro) and were 2.5–3 months old at the beginning of experiments. $PKR^{-/-}$ male mice on a 129/SvEv background (Yang et al., 1995) were obtained from Federal University of Minas Gerais Animal Centre. 129/SvEv wild-type mice were used as controls for experiments with $PKR^{-/-}$ mice. $TNFR1^{-/-}$ ($TNFRp55^{-/-}$) female mice on a C57BL/6 background (Pfeffer et al., 1993) were obtained from the University of Campinas Breeding Centre and were 3 months old at the beginning of experiments. C57BL/6 wild-type mice were used as controls for experiments with $TNFR1^{-/-}$ mice. APPSwe/PS1ΔE9 mice on a C57BL/6 background (Jankowsky et al., 2001) were obtained from The Jackson Laboratory. Wild-type littermates were used as controls. Animals were genotyped prior to the studies using specific primers (see Supplemental Experimental Procedures). All procedures involving transgenic mice were approved by the Institutional Animal Care and Use Committee of the Federal University of Rio de Janeiro (protocol #IBqM 055). Animals were housed in groups of five in each cage with free access to food and water, with controlled room temperature and humidity, and under a 12 hr light/12 hr dark cycle. All procedures used in the present study followed the "Principles of Laboratory Animal Care" from the US National Institutes of Health.

Intracerebroventricular Injections in Mice

For i.c.v. injection of AβOs, animals were anesthetized for 7 min with 2.5% isoflurane (Cristália) using a vaporizer system and gently restrained only during the injection procedure itself, as described (Figueiredo et al., 2013; Ledo et al., 2013). A 2.5 mm long needle was unilaterally inserted 1 mm to the right of the midline point equidistant from each eye and 1 mm posterior to a line drawn through the anterior base of the eye (Ledo et al., 2013). AβOs (10 pmol), 1 μg of thapsigargin, or vehicle was injected in a final volume of 3 μl, and the needle was kept in place for 30 s to avoid backflow. Before euthanasia, blue staining was injected using the same hole used previously, which allowed us to determine the accurate placement of the injection. Behavioral results from mice that showed signs of misplaced injections or any sign of hemorrhage were excluded from the final statistical analysis (this happened in 5% of cases, on average).

In Vivo Drug Treatments in Mice

Exendin-4 and liraglutide are two long-lasting GLP-1 receptor agonists. APPSwe/PS1ΔE9 mice (13–14 months old) received daily intraperitoneal injections of exendin-4 (25 nmol/kg, dissolved in saline) or vehicle (saline) for 3 weeks. Another group of transgenic animals received daily intraperitoneal injections of liraglutide (25 nmol/kg, dissolved in saline) or vehicle (saline) for 6 weeks. Male Swiss mice (3 months old) received daily intraperitoneal injections of infliximab (20 μg/day) for 7 days starting immediately after i.c.v. injection of AβOs. In experiments with 4-phenylbutyrate (4-PBA), one intraperitoneal injection (200 mg/kg) was given immediately after i.c.v injection of either Aβ oligomers or thapsigargin in male Swiss mice. 4-PBA solutions were prepared as described. Salubrinal (1 mg/kg) or vehicle was administered intraperitoneally in 3-month-old male Swiss mice for 7 days before cognitive analysis (Moreno et al., 2012). All procedures were approved by the Institutional Animal Care and Use Committee of the Federal University of Rio de Janeiro (protocol #IBqM 041).

Intracerebroventricular Injection of Aβ Oligomers in Monkeys and Treatment with Liraglutide

Adult cynomolgus monkeys (*Macaca fascicularis*) (n = 9) were used (body weights 4.7–7.0 kg). All procedures were approved by the Queen's University Animal Care Committee and were in full compliance with the Canada Council on Animal Care (Animal Care Protocol Original Munoz-2011-039-Or). Animals were under the close supervision of an animal technician and the institute veterinarian. AβOs were infused chronically through an intracerebroventricular cannula. A total of 4 animals received 100 μg Aβ oligomers i.c.v. every 3 days for 24 days, while 3 animals served as sham-operated controls. Control animals had the cannula implanted into the lateral ventricle in the same manner as the experimental animals but did not receive oligomer injections. Two additional animals received daily subcutaneous injections of liraglutide (25 nmol/kg) beginning 1 week prior to Aβ oligomer infusion and continuing until the end of AβO injections. Oligomers were freshly prepared and characterized by size-exclusion chromatography before each injection. Upon completion of the experimental protocol, animals were sedated with ketamine (10 mg/kg, intramuscular) plus buprenorphine (0.01 mg/kg) for analgesia, followed by intravenous sodium pentobarbital (25 mg/kg). Next, animals were perfused with PBS and then, sequentially, by 4% paraformaldehyde in PBS, 4% paraformaldehyde in PBS containing 2.5% glycerol, PBS + 5% glycerol, and PBS + 10% glycerol. Serial brain sections (40 μm thick) were obtained, and neuropathological analyses were performed.

Statistical Analysis

All analyses were performed with GraphPad Prism, and data sets were assessed for normality parameters prior to significance determination. Values are expressed as means ± SEM, unless otherwise stated. Statistical confidence levels are indicated in each figure (*$p < 0.05$; **$p < 0.01$).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2013.11.002.

AUTHOR CONTRIBUTIONS

M.V.L., D.P.M., S.T.F., and F.G.D.F. designed the study. M.V.L., J.R.C., R.L.F., T.R.B., L.F.-G., A.F.B., J.B.-M., L.F.-C., C.A.S., L.B.S., and S.E.-S. performed the research. M.V.L., T.R.B., C.A.S., L.F.-C., P.C.-C., O.B.A., S.T.F., and F.G.D.F. analyzed data. J.B.C., L.A.V., A.M.S., W.L.K., and C.H. contributed animals, reagents, materials, and analysis tools. M.V.L., J.-C.H., D.P.M., S.T.F., and F.G.D.F. analyzed and discussed results. M.V.L., S.T.F., and F.G.D.F. wrote the manuscript.

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