AMP-activated kinase (AMPK) is a key player in energy sensing and metabolic reprogramming under cellular energy restriction. Several studies have linked impaired AMPK function to peripheral metabolic diseases such as diabetes. However, the impact of neurological disorders, such as Alzheimer disease (AD), on AMPK function and downstream effects of altered AMPK activity on neuronal metabolism have been investigated only recently. Here, we report the impact of Aβ oligomers (AβOs), synaptotoxins that accumulate in AD brains, on neuronal AMPK activity. Short-term exposure of cultured rat hippocampal neurons or ex vivo human cortical slices to AβOs transiently decreased intracellular ATP levels and AMPK activity, as evaluated by its phosphorylation at threonine residue 172 (AMPK-Thr(P)172). The AβO-dependent reduction in AMPK-Thr(P)172 levels was mediated by glutamate receptors of the N-methyl-d-aspartate (NMDA) subtype and resulted in removal of glucose transporters (GLUTs) from the surfaces of dendritic processes in hippocampal neurons. Importantly, insulin prevented the AβO-induced inhibition of AMPK. Our results establish a novel toxic impact of AβOs on neuronal metabolism and suggest that AβO-induced, NMDA receptor-mediated AMPK inhibition may play a key role in early brain metabolic defects in AD.

AMP-activated kinase (AMPK) is a major sensor of cellular energy status and a central regulator of metabolic homeostasis (1). AMPK is activated by elevated intracellular AMP/ATP ratios in response to nutrient deprivation and/or pathological stress (1, 2), and acts pleiotropically to promote metabolic reprogramming (1, 3). Defects in AMPK signaling have been reported in peripheral metabolic disorders (4, 5), and recent evidence suggests that impaired AMPK function may play relevant roles in brain disease, such as Huntington (HD), Parkinson, and Alzheimer (AD) diseases (6). AMPK is highly expressed in the hippocampus (7), a brain region that plays key roles in synaptic plasticity, memory, and cognition, and aberrant AMPK activity has been reported in the brains of transgenic mouse models of AD (8–10) and AD patients (9, 11).

Because AD patients exhibit impaired brain metabolism (12, 13) and AMPK is required for proper metabolism and cell function (2, 3), we aimed to determine the impact of Aβ oligomers (AβOs) on neuronal AMPK. AβOs are toxins that build up in AD brains (14, 15) and in the brains of animal models of AD (16, 17), and are thought to underlie synapse and memory failure (18, 19).

Using in vitro and ex vivo models, we found that AβOs transiently inhibit neuronal AMPK phosphorylation via an NMDA receptor (NMDAR)-mediated mechanism. We further found that AβOs instigate removal of glucose transporters 3 and 4 (GLUT3 and GLUT4, respectively) from the plasma membrane in hippocampal neurons via impaired AMPK activity. AβO-induced AMPK inhibition is accompanied by depletion of intracellular ATP in neurons. Significantly, the metabolic impact initiated by oligomers could be prevented by application of insulin. Current findings demonstrate a deleterious impact of AβOs on AMPK activity that may contribute to initial brain metabolic dysfunction, and suggest that maintaining physiological AMPK activity may be a useful approach to prevent neuronal damage in AD.

**Results**

AβOs reduce AMPK-Thr(P)172 in cultured hippocampal neurons and ex vivo human cortical slices

AMPK is a heterotrimeric enzyme comprised of a catalytic (α1 or α2) and two regulatory (β1 or β2, and γ1, γ2, or γ3) subunits. Binding of AMP to the γ subunit leads to AMPK activation by the obligatory phosphorylation at Thr172 (AMPK-
**Aβ oligomers transiently inhibit neuronal AMPK**

Thr(P)\textsuperscript{172} (2, 3); hence, AMPK-Thr(P)\textsuperscript{172} is routinely used as a proxy for AMPK activity (9, 20). We initially investigated the impact of AβOs on AMPK-Thr(P)\textsuperscript{172} or total AMPK levels by immunocytochemistry in mature hippocampal neuronal cultures. AMPK-Thr(P)\textsuperscript{172} levels were significantly decreased in neurons exposed to 500 nM AβOs for 3 h (Fig. 1, A–C). Scrambled Aβ peptide had no effect on AMPK-Thr(P)\textsuperscript{172} levels (Fig. 1C). Total AMPK levels were not affected by 3-h exposure to AβOs (Fig. 1, D–F). We further investigated the impact of AβOs on neuronal AMPK-Thr(P)\textsuperscript{172} levels by Western immunoblotting. Results confirmed that AMPK-Thr(P)\textsuperscript{172} levels were reduced in cultures exposed to AβOs for 3 or 12 h (Fig. 1, G and H). Decreased AMPK-Thr(P)\textsuperscript{172} levels were further verified in *ex vivo* human cortical slices exposed to AβOs for 12 h (Fig. 1I). Results indicate that short-term AβO exposure inhibits AMPK in neurons.

**AβOs decrease ATP levels and F\textsubscript{0}F\textsubscript{1}-ATPase activity in cultured cortical neurons and in *ex vivo* human cortical slices**

Deregulation of AMPK activity is often followed by metabolic defects. To investigate whether this might take place in an AD context, we measured levels of adenine nucleotides in neurons exposed to 500 nM AβOs. Significant decreases in ATP levels, in line with our previous results (21), but not in AMP levels, were detected in both cultured cortical neurons and *ex vivo* human cortical slices exposed to AβOs for 12 h (Fig. 2, A and B). Although the ATPase activity of F\textsubscript{0}F\textsubscript{1}-ATP synthase was inhibited in neuronal cultures exposed to AβOs for 3 or 12 h (Fig. 2, C and D), the ATP synthase activity of isolated brain mitochondria was not affected by direct AβO exposure (Fig. 2E). This rules out a direct action on mitochondria and suggests that inhibition of neuronal ATP synthase was due to aberrant intracellular signaling triggered by AβOs.

![Figure 1. Short-term exposure to AβOs inhibits neuronal AMPK.](image-url)

*Figure 1. Short-term exposure to AβOs inhibits neuronal AMPK.* A–F, hippocampal cultures were exposed to vehicle (A and D), 500 nM AβOs (B and E), or scrambled Aβ (scrAβ) for 3 h and AMPK-Thr(P)\textsuperscript{172} or total AMPK levels were determined. C and F, scale bar = 10 μm. Integrated AMPK-Thr(P)\textsuperscript{172} (C) or total AMPK (F) immunofluorescence levels. Individual symbols represent mean AMPK-Thr(P)\textsuperscript{172} or total AMPK immunoreactivities from 2 to 4 coverslips for each independent culture used. Horizontal and vertical lines represent mean ± S.E. (****, *p* < 0.0001; ***, *p* < 0.05; ANOVA followed by Tukey’s test). G and H, Western blot analysis of AMPK-Thr(P)\textsuperscript{172} levels in hippocampal cultures exposed to vehicle or 500 nM AβOs for 3 or 12 h, respectively. Lanes were run on the same gel but were noncontiguous. Graphs show densitometric analysis for AMPK-Thr(P)\textsuperscript{172} normalized by total AMPK levels. Symbols represent densitometric analysis for each independent culture used. Horizontal and vertical lines represent mean ± S.E. (***, *p* < 0.001 or *, *p* < 0.05; Student’s *t* test, compared with vehicle-treated cultures). I, AMPK-Thr(P)\textsuperscript{172} levels in *ex vivo* human cortical slices exposed to vehicle or 500 nM AβOs for 12 h. Graphs show densitometric analysis for AMPK-Thr(P)\textsuperscript{172} normalized by total AMPK levels. Symbols represent densitometric analysis for each independent human cortical slice culture used. Horizontal and vertical lines represent mean ± S.E. (p = 0.063; Student’s *t* test, compared with vehicle-treated cultures).
Besides impaired mitochondrial synthesis of ATP, its extracellular release and catabolism to adenosine could also contribute to decreased ATP levels in cultured neurons under stress (22). Indeed, significant increases in extracellular adenosine were detected in neuronal cultures exposed to AβOs for 3 or 12 h (Fig. 2, F and G). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction measurements showed no inhibition of cellular redox activity in cultures exposed to AβOs for 12 h (Fig. 2H), ruling out the possibility that increased extracellular adenosine levels were due to release from dead or damaged cells. These results indicate that AβO-exposed neurons undergo disturbances in energy homeostasis that include inhibition of mitochondrial synthesis of ATP and stimulation of its extracellular release.

**NMDA receptors mediate the decrease in AMPK-Thr(P)172 in cultured hippocampal neurons**

In previous works, we have demonstrated that AβOs cause activation of neuronal NMDARs leading to a rapid increase in intracellular calcium levels and causing abnormal neuronal accumulation of reactive oxygen species, among other pathological consequences (23–26). We thus hypothesized that activation of NMDARs and calcium-dependent protein phosphatase calcineurin/protein phosphatase 2B could mediate the
Aβ oligomers transiently inhibit neuronal AMPK

Impact of AβOs on AMPK-Thr(P)172 levels. To test this possibility, hippocampal cultures were pre-treated with the NMDAR blocker, MK801, or the calcineurin inhibitor, FK506, prior to the 3-h exposure to AβOs. Results indicate that MK801 prevented the impact of oligomers on AMPK-Thr(P)172, whereas no effect was observed with FK506 (Fig. 3, A and B). These observations suggest that NMDARs, but not calcineurin, are implicated in oligomer-induced AMPK inhibition.

Pharmacological stimulation of AMPK counteracts its inhibition by AβOs

If AβO-induced reductions in AMPK-Thr(P)172 levels indeed mirror alterations in its activity, one might expect that pharmacological agents known to stimulate AMPK activity should be able to counteract AβO actions. In line with this hypothesis, pre-treatment with AICAR, an AMP-mimicking riboside, or with metformin, a known AMPK activator, prevented the decrease in AMPK-Thr(P)172 levels caused by exposure to AβOs in hippocampal neurons (Fig. 4).

AβOs decrease surface levels of dendritic GLUT3 and GLUT4 in hippocampal neurons

AMPK plays a central role in cellular glucose uptake by facilitating the translocation of glucose transporters to the plasma membrane (27, 28). Because levels of GLUT3 and GLUT4 are decreased in AD brains (29, 30), we investigated the impact of AβOs on surface levels of these transporters in hippocampal cultures. Neurons targeted by AβOs exhibited markedly decreased surface levels of dendritic GLUT3 (Fig. 5, A–G) and GLUT4 (Fig. 5, H–N). As expected, compound C, a pharmacological inhibitor of AMPK, decreased dendritic surface GLUT3 and GLUT4 levels (Fig. 5, E and L). Interestingly, AICAR blocked the decrease in surface levels of GLUTs induced by AβOs (Fig. 5, E and L). Results indicate that AβO-induced decreases in surface levels of glucose transporters are mediated by AMPK inhibition in neurons.

Longer term exposure to AβOs does not trigger prolonged metabolic impairment or neuronal death

Because prolonged inhibition of AMPK and sustained reductions in ATP and surface GLUT levels could lead to decreased cell viability and cell death, we investigated whether the impact of AβOs on neuronal metabolism would persist upon longer term exposures. We found that levels of AMPK-Thr(P)172 (Fig. 6A), total AMPK (Fig. 6B), and intracellular ATP (Fig. 6C) in neurons exposed to AβOs for 24 h were similar to those in vehicle-treated cultures.

To examine the possibility that restoration of AMPK-Thr(P)172 levels after 24 h of exposure to oligomers was due to reduced levels of AβOs in the culture medium, we aimed to determine whether extracellular AβO levels were maintained for the duration of the 24-h incubation period. We exposed neuronal cultures to 500 nM AβOs for 3 or 24 h. Culture medium from 3 independent cultures was then collected, and immunoblot dot analysis was carried out using the NU4 anti-oligomer monoclonal antibody (31). Similar AβO immunoreactivities were observed at 3 or 24 h (compared with 500 nM AβOs in Neurobasal medium alone, used as a control), suggesting that restored levels of AMPK-Thr(P)172 were not related to depletion of AβOs from the medium upon longer (24 h) incubation with neuronal cultures (Fig. 6D). We further found that oligomers remain bound to neuronal processes 24 h after addition of AβOs to cultures (Fig. 6E), with a binding pattern identical to that observed at 3 h (Fig. 5, D and K).

Furthermore, no significant impact on surface levels of GLUT3 (Fig. 6, F and G) was verified after 24 h of exposure of neurons to AβOs. A nonstatistically significant (p = 0.08) trend of decrease in surface levels of GLUT4 was observed after 24 h of exposure to AβOs (Fig. 6, H and I).

Control experiments showed that exposure to 500 nM AβOs for 12, 24, or 48 h had no effect on MTT reduction in hippocampal cultures (Fig. 6I; see also Ref. 32), indicating preserved cellular redox activity under these conditions. No reduction in cell viability was further detected in hippocampal neurons exposed to AβOs or scrambled Aβ for 24 or 48 h, as measured using a live/dead assay (Fig. 6K). Collectively, results indicate that AβOs trigger transient inhibition of AMPK with early deleterious consequences in terms of neuronal energy metabolism (decreased ATP and surface levels of GLUTs), and that compensatory mechanisms operate to re-establish homeostasis and prevent further metabolic damage and cell death upon longer term exposure to AβOs.
Insulin prevents AβO-induced AMPK inhibition and adenosine release in hippocampal neurons

Stimulation of brain insulin signaling has been suggested as a promising therapeutic approach in AD (33–35). We have previously shown that insulin prevents inhibition of brain insulin signaling, activation of aberrant cellular stress mechanisms, and synapse deterioration induced by AβOs (36–38). We thus hypothesized that bolstering insulin signaling might protect neurons from the inhibition of AMPK by AβOs.

We found that pre-treatment of hippocampal cultures with insulin prevented the decrease in neuronal AMPK-Thr(P)172 levels (Fig. 7, A and B) induced by a 3-h exposure to AβOs. Insulin alone had no effect on neuronal AMPK-Thr(P)172 levels (Fig. 7B). Insulin further prevented the increase in extracellular adenosine levels induced by 3- or 12-h exposure of hippocampal cultures to AβOs (Fig. 7, C and D). Results indicate that insulin prevents the inhibition of AMPK and metabolic deregulation induced by AβOs, thereby extending the grounds toward the translational potential of insulin in AD.

Discussion

Using in vitro and ex vivo models of AD, we have found that AβOs transiently inhibit neuronal AMPK through a NMDA receptor-dependent mechanism, and decrease ATP and glucose transporter levels. Previous observations indicate that AβOs are implicated in different processes leading to neuronal dysfunction, including oxidative stress, abnormal Tau phosphorylation, and synapse loss (32, 39–42). The discovery that AβOs interfere with AMPK activity indicates a new mechanism contributing to neuronal metabolic defects that are germane to AD.

AMPK has been implicated in other brain disorders, including stroke (43), HD (44), and tauopathies (11). Overactivation of AMPK was reported in the striatum of Huntington patients, and further implicated in brain atrophy, neuronal loss, and formation of huntingtin aggregates in a HD transgenic mouse model (45). Thus, altered AMPK may comprise a common feature leading to impaired neuronal metabolic function in neurodegenerative diseases.

Our findings are in line with recent reports suggesting that soluble Aβ species reduce neuronal AMPK-Thr(P)172 in vitro (20, 46). On the other hand, previous studies have shown that Aβ rapidly and transiently (from 10 to 60 min) stimulates AMPK-Thr(P)172 in cultured neurons (20, 47). At first sight, such an oscillating behavior of AMPK-Thr(P)172 could be interpreted as conflicting observations in the literature. Nevertheless, both AMPK activation within minutes and the subsequent longer term inhibition induced by Aβ have been linked to increased Tau phosphorylation and attenuated protein synthesis in neuronal cultures through distinct mechanisms (20, 46, 47).

Our current results indicate preserved cell viability even after a 24-h exposure to AβOs. Therefore, reductions in AMPK...
Aβ oligomers transiently inhibit neuronal AMPK

Figure 5. AβOs induce short-term reductions in surface levels of GLUT3 and GLUT4 in cultured hippocampal neurons. A–G, GLUT3 immunofluorescence (green) in hippocampal cultures exposed to vehicle (A) or 500 nm AβOs (C) for 3 h. B and D, AβO immunolabeling (red; NU4 antibody). F and G, merged double-labeled images of vehicle- or AβO-exposed cultures, respectively. E, quantitative analysis of surface levels of GLUT3 from experiments with 2–5 independent cultures. Symbols represent mean GLUT3 immunoreactivities from 2 to 4 coverslips for each independent culture used. H–N, GLUT4 immunofluorescence (green) in hippocampal cultures exposed to vehicle (H) or 500 nm AβOs (I) for 3 h. J and K, AβO immunolabeling (NU4 antibody; red). M and N, merged double-labeled images of vehicle- or AβO-exposed cultures, respectively. K, quantitative analysis of surface levels of GLUT4 from experiments with 2–3 independent cultures. Symbols represent mean GLUT4 immunoreactivities from 2 to 3 coverslips for each independent culture used. Horizontal and vertical lines represent mean ± S.E. (**, p < 0.005; ***p < 0.0001; one-way ANOVA followed by Bonferroni post hoc test compared with vehicle-treated cultures). Scale bar = 10 μm.

activity reported here likely reflect aberrant signaling mechanisms rather than cell death. We further provide initial insight into the mechanism underlying transient AMPK inhibition following exposure to AβOs. We show that it involves signaling via NMDA receptors, whereas calcineurin does not appear to play a significant role in this process. Overactivation of NMDARs has been shown to mediate oligomer-induced neuronal defects, including oxidative stress and synapse loss (23–26, 48). Deregulation of intracellular calcium homeostasis initiated by AβO-NMDAR signaling could lead to aberrant activation of the calcium-dependent phosphatase calcineurin/protein phosphatase 2B (49–51). It is thus possible that NMDAR-triggered pathways alter the kinase-phosphatase balance, ultimately leading to reduced AMPK activity and early impairments in neuronal metabolism. Future studies are warranted to fully elucidate the intracellular mechanisms linking AβO/NMDAR signaling to impaired AMPK and defective energy metabolism.

We found that neurons targeted by AβOs present an acute reduction in surface levels of GLUT3 and GLUT4, suggestive of a short-term metabolic defect. Interestingly, pharmacological activation of AMPK prevents the impact of AβOs on GLUTs. AβO-induced AMPK inhibition is further accompanied by decreased intracellular levels of ATP. These results add novel components to the metabolic stress landscape mediated by AMPK-Thr(P)172 fluctuations across AD progression. In this regard, an interesting recent study identified GLUT4 as a key metabolic modulatory system that up-regulates glycolysis in firing neurons to meet activity-driven ATP demand at nerve terminals (52). Significantly, AMPK was found to drive GLUT4 regulatory activity, essential for sustained synaptic transmission. It is conceivable, therefore, that AβO-induced AMPK inhibition and reduction in surface levels of neuronal GLUT4 comprises a mechanism leading to reduced or defective synapse function in AD.

Abnormal ATP release to the extracellular medium in neuronal cultures exposed to AβOs may be an important mediator of neurotoxicity via activation of purinergic receptors in AD brains. Indeed, a recent report demonstrated that AβO-induced ATP leakage activates P2X receptors to cause excitotoxicity (53). Furthermore, adenosine resulting from extracellular ATP degradation could also serve as a messenger mediating communication between neurons and glia, likely via activation of glial purinergic receptors, to exacerbate brain and memory dysfunction, as suggested (54–57).

In line with neuropathology studies in AD (9, 11) and in transgenic models of AD (8–10, 58), experimental evidence suggests a role for chronic AMPK activation in synapse dysfunction in AD transgenic models (20, 58). Our findings in neuronal cultures establish that AβOs cause early inhibition of AMPK activity, but this impact is reversed in longer time frames (24 h). Previous studies by our group and others have indicated that the neuronal impact of AβOs persist after several hours or even days of exposure of neurons to oligomers (38, 40, 59–61). Our current results suggest that, despite restored AMPK activity, AβOs remain bound to neurons after 24 h, and
no considerable signal decay is observed in oligomer binding to neurons at 3 \textit{versus} 24 h. These findings suggest the recovery in AMPK activity is not related to reduced levels of A\textsubscript{β}/H9252Os in the medium or desensitization of A\textsubscript{β}/H9252O-activated signaling pathways. On the other hand, current results show a sharp decrease in ATP levels in both rodent and human cortical neurons exposed to A\textsubscript{β}Os for 12 h. As a key cellular energy sensor, AMPK is activated in response to such a decrease in energy status. We feel this homeostatic response to reduced energy levels is responsible for the recovery of AMPK activity observed 24 h following application of A\textsubscript{β}Os. It is, thus, possible that accumulation of A\textsubscript{β}Os in pre-AD brains initially inhibits AMPK activ-
Aβ oligomers transiently inhibit neuronal AMPK

Figure 7. Insulin prevents AβO-induced AMPK inhibition and adenosine release in hippocampal cultures. A, hippocampal cultures were exposed to vehicle or 500 nM AβOs (in the absence or presence of insulin) for 3 h, and AMPK-Thr(P)172 levels were evaluated by immunocytochemistry. Scale bar = 10 μm. B, integrated AMPK-Thr(P)172 immunofluorescence levels. Insulin alone had no effect on AMPK-Thr(P)172 levels. Symbols represent mean AMPK-Thr(P)172 immunoreactivities from 2 to 4 coverslips for each independent culture used. Horizontal and vertical lines represent mean ± S.E. (*, p < 0.05; **, p < 0.001; one-way ANOVA followed by Bonferroni test). C and D, extracellular adenosine levels in hippocampal cultures exposed for 3 (C) or 12 (D) h to 500 nM AβOs, in the absence or presence of insulin (1 μM). Symbols represent analyses for each independent culture used. Horizontal and vertical lines represent mean ± S.E. (*, p < 0.05, one-way ANOVA followed by Newman-Keuls test).

It is, triggering early brain and cognitive damage. As disease progresses, however, homeostatic mechanisms may lead to persistent and excessive brain AMPK activity that could, in turn, further compromise synapse integrity and function.

The existence of compensatory mechanisms to control AMPK activity finds support in that pharmacological manipulation of brain AMPK shows only transient and limited action in promoting benefits in mice (62). Taken together, these findings suggest that the prolonged action of a homeostatic mechanism targeting AMPK function could lead to its overactivity and drive brain damage in AD. Preventing initial AMPK deregulation could thus be an approach to avoid extensive brain damage in AD.

Bolstering brain insulin signaling has been suggested as a promising therapeutic approach in AD (35, 63–65). Intranasal insulin treatment improves memory performance in early AD patients (33, 34). Furthermore, insulin has been found to protect against synapse damage and neurotoxicity induced by AβOs (36–38). Current results indicate a beneficial action of insulin against AβO-induced AMPK inhibition, extending the grounds supporting neuroprotection by insulin in animal models and human brains. Although we acknowledge that physiological concentrations of insulin would be lower than the micromolar range used in our study, neuroprotection by micro-molar concentrations of insulin has also been verified in several other experimental approaches and conditions (22, 36–38, 66, 67). We note, however, that we cannot exclude the possibility that insulin activates related receptors, including IGF-1R, at the concentration used in our study. Pharmacological distinction between possible neuroprotective actions of insulin via insulin or IGF-1 receptors is made difficult by the lack of drugs that specifically target only one of these receptors. Further complicating this issue, insulin and IGF-1 receptors may form hybrid dimers (68, 69). Nonetheless, our findings may help to explain the neuroprotective actions of insulin in the context of metabolic brain dysfunction in neurodegenerative diseases (70, 71). Further investigation of the mechanisms of insulin action in the brain will provide a more complete view on neuroprotection by insulin, notably when such information is combined with results from ongoing clinical trials.

In conclusion, our current results establish that Aβ oligomers transiently reduce neuronal AMPK activity in an NMDAR-mediated mechanism that leads to reduced surface exposure of GLUT3 and GLUT4. In addition to previously reported AMPK signaling defects in neurons (9, 11, 20, 58), impaired energy sensing and adequate metabolic shift could render neurons more vulnerable to stress, as recently pointed out (72). Furthermore, the recent demonstration that activity-dependent, AMPK-mediated synaptic GLUT4 mobilization is essential for synapse function (52) suggests a novel mechanism by which AβOs may lead to synapse dysfunction. Thus, the effects of AβOs on AMPK may contribute to AD-linked brain metabolic and synaptic defects. AMPK is a key regulator of metabolic homeostasis and therefore could be a target for novel therapeutic opportunities in AD.

Experimental procedures

Materials

Synthetic Aβ(1–42) peptide was from American Peptide (Sunnyvale, CA). Culture media/reagents, Alexa Fluor-labeled secondary antibodies, and ProLong anti-fade reagent were from Invitrogen. Fluorescent secondary antibodies for Western blotting were from LiCor (Lincoln, NE). Antibodies against
Aβ oligomers

AβOs were prepared from synthetic Aβ(1–42) as previously described (32, 37). Oligomer preparations were routinely characterized by size exclusion HPLC and, occasionally, by Western blots using oligomer-sensitive NU4 monoclonal antibody (31), and comprised a mixture of Aβ dimers, trimers, tetramers, and higher molecular weight oligomers. Protein concentration was determined using the BCA assay (Thermo-Pierce).

Mature cortical and hippocampal neuronal cultures

Cortices from 14-day-old rat embryos or hippocampi from 18-day-old embryos were dissected and cultured as previously described (73, 74) with minor modifications. Briefly, dissociated cells were suspended in Neurobasal medium supplemented with B27, Glutamax, and antibiotics, and were plated on glass coverslips previously coated with 1.5 μg/ml of poly-L-lysine. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂ for 18–21 days prior to use. When present, insulin (1 μM), MK-801 (10 μM), AICAR (2.5 mM), compound C (10 μM), or metformin (2 mM) were added to cultures 30 min before application of vehicle or AβOs (at 250 or 500 nM, as indicated under “Results”).

Ex vivo human cortical slices

Adult human cortical slices were prepared as described (75). Healthy cortical tissue was obtained from patients with drug-resistant epilepsy subjected to temporal lobectomy for removal of hippocampal epileptic foci. After dissection under sterile conditions, 0.4-mm thick slices were prepared using a McIlwain tissue chopper and maintained in supplemented Neurobasal medium. Alexa Fluor-conjugated secondary antibodies were used. Coverslips were imaged on a Nikon Eclipse TE 2000-U fluorescence microscope (for AMPK-Thr (P)172 and AMPK labeling) and Zeiss Axio Observer Z1 microscope (for GLUT imaging). Omission of primary antibodies eliminated all labeling.

Measurement of intracellular adenine nucleotide levels

Adenine nucleotide contents in 18–21-day-old in vitro rat cortical neurons were analyzed by ion-paired reverse phase liquid chromatography. Cultures were exposed to vehicle, 500 nM AβOs, or insulin + AβOs for 12 h. When present, insulin (1 μM) was added to cultures 30 min before AβOs. The culture medium was removed and liquid nitrogen was used to disrupt cells and stop cellular metabolism. The plates were kept in an ice bath until the liquid nitrogen evaporated completely. Cells were homogenized and proteins were precipitated with 6% trichloroacetic acid, and the suspension was neutralized by adding a small aliquot of 1 M Tris solution and centrifuged at 20,800 × g for 5 min at 4 °C. Protein content was determined using the BCA assay. Aliquots were injected into an HPLC system using a Supelguard column (Supelco) coupled to a Super- cosil C-18 carrier (particle size 5 μm, Supelco) column. Isoocratic elution was performed at a flow-rate of 1 ml/min at room temperature with 50 mM KH₂PO₄ buffer, pH 6, methanol (90/10), and 4 mM tetrabutylammonium bromide. Nucleotide levels were measured by UV absorbance at 254 nm (ε = 15,400 M⁻¹ cm⁻¹). ATP, ADP, AMP, and adenosine peaks were identified by co-injecting 2-nmol standards in an independent run. Results were expressed by normalizing peak areas by the total amount of protein.

Western and dot immunoblotting

Cultures were harvested with RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, and 1% Triton X-100) plus protease/phosphatase inhibitors. Lysates were incubated on ice for 30 min with 5-s vortexing every 5 min and were centrifuged

pAMPK (Thr¹⁷²) and AMPK were from Santa Cruz Biotechnology (Santa Cruz, CA) or Cell Signaling (Danvers, MA). Antibodies against GLUT3, GLUT4, and β-actin were from Abcam. The oligomer-selective antibody NU4 (31) was produced in Dr. Klein’s laboratory. SuperSignal West Femto Maximum Sensitivity substrate, protease/phosphatase inhibitors were from Thermo-Pierce (Rockford, IL). Tetrabutylammonium bromide was from Fluka (Steinheim/Switzerland). Metformin (1,1-dimethylbiguanide hydrochloride), compound C, and AICA-riboside were from Calbiochem (Darmstadt, Germany). Insulin was from Eli Lilly (Indianapolis, IL). FK506 was from Tocris Biosciences (Bristol, UK). MK801 was from Sigma.

Aβ oligomers transiently inhibit neuronal AMPK

with 2 μM calcein and 1 μM ethidium. Cells were imaged on a Nikon Eclipse TE 2000-U fluorescence microscope and the proportion of live (calcein-positive) or dead (ethidium-positive) cells were determined relative to the total number of cells. Three experiments with independent neuronal cultures were performed.

The MTT reduction assay (Roche Applied Science) was used to evaluate cellular metabolic redox activity in 18-day old cortical neuronal cultures. Three experiments with independent neuronal cultures (each using 3 wells per experimental condition) were carried out as described (75).

Immunocytochemistry

Cells were fixed and blocked as described previously (37) and probed with anti-pThr¹⁷²-AMPK (1:250), anti-AMPK (1:100) and oligomer-sensitive NU4 antibody (31). For immunodetection of surface-exposed GLUT3 or GLUT4, cells were double-labeled with oligomer-sensitive NU4 mouse monoclonal antibody and either GLUT3 (Abcam, 1:750) or GLUT4 (Abcam, 1:500) rabbit polyclonal antibodies under non-permeabilizing conditions. Alexa Fluor-conjugated secondary antibodies were used. Coverslips were imaged on a Nikon Eclipse TE 2000-U fluorescence microscope (for AMPK-Thr(P)¹⁷² and AMPK labeling) and Zeiss Axio Observer Z1 microscope (for GLUT imaging). Omission of primary antibodies eliminated all labeling.
Aβ oligomers transiently inhibit neuronal AMPK

at 10,000 × g for 5 min at 4 °C. Protein concentration in the supernatant was determined by the BCA assay. Thirty micrograms of protein were resolved on 10% SDS-PAGE and analyzed by Western blot using anti-pThr172-AMPK (1:500) or anti-AMPKα1–2 (1:2,000) antibodies. β-Actin (1:50,000) was used as a loading control. For dot immunoblot analyses, culture media were removed from hippocampal cultures after treatments and immediately frozen until analyses. Samples (4 μl) were spotted onto nitrocellulose membranes and incubated in blocking solution (3% BSA) for 1 h. Membranes were then incubated with the oligomer-selective antibody NU4 (31) (1:1,000) overnight. Immunoblots were developed either by chemiluminescence using HRP-conjugated secondary antibodies (1:20,000), or by fluorescence using infrared dye-conjugated secondary antibodies (1:5,000–1:10,000; LiCor) for Odyssey.

F,F₁-ATPase activity

Cortical cultures were exposed to 500 nm AβOs or vehicle (2% DMSO in PBS) for 3 or 12 h (as indicated under “Results”). Cultures were washed twice and lysed in ice-cold PBS. Lysates were centrifuged at 14,000 rpm for 5 min at 4 °C. The supernatant was discarded and the pellet was resuspended in buffer containing 0.32 m sucrose, 1 mM EDTA, 1 mM EGTA, and 10 mM Tris-HCl, pH 7.0, 4 mM MgCl₂, and 1 mM ATP. The mitochondrial F₀-F₁-ATPase is the difference between activities in the presence and absence of azide, and is referred to as azide-sensitive ATPase. Inorganic phosphate (Pᵢ) derived from ATP hydrolysis was determined by a colorimetric method with minor modifications (76).

Image analysis

AMPK-Thr(P)¹⁷², total AMPK, GLUT3, and GLUT4 immunofluorescence intensities were analyzed in a total of 3–6 experiments (see figure legends) using independent neuronal cultures. Immunofluorescence of each neuron, including cell bodies and dendrites, was quantified in each experimental condition. Quantitative analysis of immunofluorescence data were carried out by histogram analysis of the fluorescence intensity at each pixel across the images using Image J (NIH; Windows version). Briefly, the program analyzes a grayscale image by plots of the intensity histogram and by calculation of average intensity and standard deviation. Appropriate thresholding was employed to eliminate background signal in the images before histogram analysis, and fluorescence intensity was normalized by the number of immunoreactive cells in each field. To exclude the possibility that exposure to AβOs could lead to alterations in neuronal cell area, which could potentially bias pAMPK analyses, we determined the areas of MAP2 (used as an independent and unbiased neuronal marker that is not affected by oligomers) immunoreactive cells in four independent cultures, and found that AβOs do not cause significant variations in neuronal area across cultures (not shown). In each experiment, 20–30 images were acquired from 3 coverslips in each experimental condition. The results of the analysis of images were then combined to allow quantitative estimates of changes in neuronal levels of AMPK-Thr(P)¹⁷², total AMPK, GLUT3, or GLUT4.

Statistics

Statistical significances were assessed by Student’s t test (for comparison between two experimental conditions) or ANOVA (for multiple comparisons) followed by appropriate post hoc tests, using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). See legends for specific details on statistical analyses.

Study approval

Experiments involving hippocampal cultures were performed in certified facilities under protocols approved by the Institutional Animal Care and Use Committee of the Federal University of Rio de Janeiro (protocol number IBQM 077). All procedures involving human cortical tissue were approved and regulated by the Committee for Research Ethics of the Clementino Fraga Filho University Hospital (protocol number 0069.0.197.000-05). Donors gave written informed consent for use of brain tissue that would otherwise have been discarded.

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