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mTORC2 (Rictor) in Alzheimer's Disease and Reversal of Amyloid- β Expression-Induced Insulin Resistance and Toxicity in Rat Primary Cortical Neurons

Han-Kyu Lee^a, Bumsup Kwon^a, Cynthia A. Lemere^b, Suzanne de la Monte^c, Kyohei Itamura^a, Austin Y. Ha^a, and Henry W. Querfurth^{a,1,*}

^aDepartment of Neurology, Rhode Island Hospital and Brown University Warren Alpert Medical School, Providence, RI, USA

^bAnn Romney Center for Neurologic Diseases, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA

^cDepartment of Pathology, Rhode Island Hospital and Brown University Warren Alpert Medical School, Providence, RI, USA

Abstract

Mammalian target of rapamycin complex 1 (mTORC1), a nutrient sensor and central controller of cell growth and proliferation, is altered in various models of Alzheimer's disease (AD). Even less studied or understood in AD is mammalian target of rapamycin complex 2 (mTORC2) that influences cellular metabolism, in part through the regulations of Akt/PKB and SGK. Dysregulation of insulin/PI3K/Akt signaling is another important feature of AD pathogenesis. We found that both total mTORC1 and C2 protein levels and individual C1 and C2 enzymatic activities were decreased in human AD brain samples. In two rodent AD models, mTORC1 and C2 activities were also decreased. In a neuronal culture model of AD characterized by accumulation of cellular amyloid- β (A β)₄₂, mTORC1 activity was reduced. Autophagic vesicles and markers were correspondingly increased and new protein synthesis was inhibited, consistent with mTORC1 hypofunction. Interestingly, mTORC2 activity in neural culture seemed resistant to the effects of intracellular amyloid. In various cell lines, A β expression provoked insulin resistance, characterized by inhibition of stimulated Akt phosphorylation, and an increase in negative mTORC1 regulator, p-AMPK, itself a nutrient sensor. Rapamycin decreased phospho-mTOR and to lesser degree p-Rictor. This further suppression of mTORC1 activity protected cells from A β -induced toxicity and insulin resistance. More striking, Rictor over-expression fully reversed the A β -effects on primary neuronal cultures. Finally, using *in vitro* assay, Rictor protein addition completely overcame oligomeric A β -induced inhibition of the PDK-Akt activation step.

*Correspondence to: Henry Querfurth, MD, PhD, Department of Neurology, Rhode Island Hospital and Brown University Warren Alpert Medical School, 593 Eddy St., Providence, RI 02903, USA. Tel: +1 617 667 5553; Fax: +1 617 667 7981; henry_querfurth@brown.edu.

¹Current address: Department of Neurology, Beth Israel Deaconess Medical Center, 330 Brookline Ave. Boston, MA 02215, USA. hquerfur@bidmc.harvard.edu.

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SUPPLEMENTARY MATERIAL

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We conclude that striking a new balance by restoring mTORC2 abundance and/or inhibition of mTORC1 has therapeutic potential in AD.

Keywords

Akt; Alzheimer's disease; AMPK; autophagy; insulin resistance; mTOR; mTORC2; Rictor

INTRODUCTION

The mammalian target of rapamycin complex 1 (mTORC1), comprising Raptor (and mTOR, GβL/mLST8, and the non-core proline-rich Akt substrate of 40 kDa (PRAS40) and Deptor proteins) signal pathway is a crucial cellular energy and nutrient sensor as well as growth factor (insulin, brain-derived neurotrophic factor (BDNF)) transducer that controls downstream targets eIF-4E binding protein (4EBP1) and p70 ribosomal protein S6 kinase 1 (p70S6K) and processes such as the initiation step of mRNA to protein translation, protein synthesis, and ribosome biogenesis [1]. Accordingly, mTORC1 has been found to support protein synthesis dependent synaptic plasticity that underlies learning and long term memory such that rapamycin and various genetic manipulations block several types of memory processes such as fear conditioning and late phase-long-term potentiation (LTP) [2–4]. Inhibition of autophagy and stimulation of mitochondrial respiration are other key roles [5, 6]. Cell growth, division, survival, and aging are accordingly affected. One target of mTORC1, p70S6K, has the additional feedback role to downregulate insulin signaling at the level of insulin receptor substrate 1 (IRS-1) [7]. This function of activated mTORC1 to negatively regulate sustained PI3K/Akt activation by insulin [8, 9] has central importance to the widely held notion that the Alzheimer's disease (AD) brain is an insulin resistant organ from an inactivating phosphorylation at IRS-1 by p70S6K [10, 11]. In contrast, mTORC2 is a relatively rapamycin-resistant complex comprising Rictor (and mTOR, GβL/mLST8, mSIN1, PRR5/Protor, and Deptor) and while not regulated directly by nutrients, is insulin responsive and feeds forward to amplify the activation of the S-T kinase Akt (Protein Kinase B) by insulin/insulin-like growth factor-1 (IGF-1) in acting as an S473 kinase (PDK2) [12–16]. Other targets of mTORC2 as metabolic regulator and cell survival promoter include the actin cytoskeleton and serum/glucocorticoid-regulated kinase 1 (SGK-1) [17, 18]. A second mechanism of activated mTORC1 mediated suppression of insulin action is the inhibitory T1135 phosphorylation of Rictor [19], thereby dampening the insulin response at the level of Akt. Thus, by inhibiting mTORC1, short term rapamycin treatment may activate Akt via Rictor (and S6K suppression) whereas long term rapamycin disassembles mTORC2 causing insulin resistance [20, 21].

Evidence points to AD brain as affected by a unique form of insulin deficiency and resistance [22, 23]. In addition, autophagy is altered [11, 24]. Thus, it is important to understand what impact changes in mTOR signaling have for early disease pathogenesis. mTOR integrating functions are essential to certain observations of relevance to AD such as age extension and delay of proteotoxicity. Regarding the latter, neuroprotection in transgenic or control rodents has been achieved by: 1) downregulation of insulin/IGF-1 signaling pathway [25–28], 2) caloric restriction/Sirt1 stimulation [29,30], and 3) rapamycin

treatment [31]. Opposing evidence in favor of insulin/IGF1 mediated neuroprotection [32] has nonetheless created much discussion and debate.

Differing accounts of mTOR status in AD brain, transgenic mice, and cell models have also appeared. Several groups report dramatic upregulation of basal mTOR signaling markers in AD temporal cortex (pS2481 mTOR, p-4EBP1, p-eEF2K, p-p70S6K, and p-eIF4E). Their levels were positively correlated to neurofibrillary tangle load, total- and paired helical filament-tau burden, and excessive tau mRNA translation [33–36]. Others cite elevated pS2448 mTOR and/or mTOR expression in AD brain samples [11, 37, 38], as well as evidence for Akt activation [39–41]. In cell models, using either transgenic primary cortical neurons (PCNs) or control PCNs exposed to A β oligomers, abnormal phospho-activations of Akt (p-S473) and mTOR (pSer2448) / 4EBP1(p-S65) pathway components were associated with aberrant cell cycle reentry [42]. In a *Drosophila* tauopathy, mTOR activation was also found to mediate cell cycle reentry and neurodegeneration [43]. mTOR signaling increases are also described in 3xTgAD and PDAPP transgenic mice, where inhibition of mTOR with rapamycin rescued early learning and memory deficits and activated autophagy [44–46]. In further experiments by the same group, intra hippocampal anti-A β antibody injections normalized the abnormal mTOR activation. In their model, Akt activation was deduced to drive proline-rich Akt substrate of 40 kDa (PRAS40) phosphorylation, thereby de-repressing mTOR [45].

On the other hand, in a recent study of autopsy brain, levels of p-mTOR (Ser2448), p-mTOR (Ser2481), and total mTOR revealed no statistical differences across the clinical groups (AD versus control) [47]. In the only study of Rictor (mTORC2) we uncovered, expression levels were unaltered in AD [38]. The same study found total and phospho-mTOR and Raptor levels were equally increased only in severely affected AD brain. Results pointing to downregulation of mTOR signaling (pS2448 and p-p70S6K) were obtained in N2A cells affected by aggregated A β ₄₂, in double transgenic APP(s1)/PS1(M146L) mouse cortex and in AD lymphocytes compared to controls [48]. Moreover, APP(swe)/PS1(deltaE9) transgenic mice display increased autophagic activity accompanied by decreased mTOR activity [49]. In yet another transgenic model, APP(s1)/PS1(KI), while mTOR itself was unchanged, downstream activation of p70S6K (pT389) was reduced rather than stimulated [50]. Consistent with these, but using a growth factor stimulation paradigm in rat PCNs, A β treatment inhibited BDNF-induced, Akt-mTOR signal activation [51]. Finally, in the transgenic APPTg2576 model, decay of LTP was correlated with inhibited mTOR signaling (lowered p-p70S6K and p-4EBP1), similar to results in wild-type (wt) slices exposed to A β peptide or rapamycin [52, 53]. Upregulation of mTOR rescued LTP in this model [52]. As noted above, however, in an AD transgenic model where basal mTORC1 is abnormally over-activated or in yet another where downstream marker p-p70S6K was not, rapamycin had a beneficial effect on restoring memory formation and maintenance [44, 46]. Thus, there is a duality of mTOR roles in health and disease with respect to synaptic plasticity [54].

While some macroautophagy markers appear induced in AD, consistent with the basal suppression of mTOR found in the above studies, it appears so primarily because lysosomal clearance is reduced, resulting in a net impairment of autophagosome flux [24, 55, 56]. Yet rapamycin, by suppressing C1, induces autophagy and ameliorates cognitive deficits in mice

[57]. Genetic reduction of mTOR in Tg2576 AD mice also reduced A β pathology, stimulated autophagy and rescued memory deficits [58]. Recently, autophagy markers were found decreased in mild cognitive impairment and AD brain. These correlated negatively with amyloid load and were associated with hyperactivated PI3K/Akt/mTOR axis [11].

In considering rapamycin-like pharmacologic therapy for AD, it remains uncertain whether it will improve autophagy or alleviate other mTOR dysfunctions, particularly if mTOR is already inhibited in some AD patient populations. Notwithstanding the variance between reported results, most studies have either not directly analyzed mTOR enzymatic activity, reporting only on proxy phospho-marker levels, nor tested for insulin-stimulated mTOR changes and have not explored the role of mTORC2. Therefore, we tested these mTOR parameters in several AD models.

MATERIALS AND METHODS

Ethics statement

The study was approved by the Institutional Ethics Committee and was based on a project entitled; “Clinical, biochemical, electrophysiologic evaluation of normal transgenic and knock-out mice relevant to neurodegenerative disease of brain and skeletal muscle (462439-3/2013).”

Preparation of frozen brain tissue for biochemical analysis

AD brain samples, prefrontal and temporal cortex excluding white matter, Braak stages V-VI, were obtained from the Harvard Brain Tissue Resource Center, McLean Hospital and extracted into lysis buffer. From a total pool of 20 patient and 20 control samples, 19 each were tested by PAGE and immunoblot for mTOR and p-mTOR (Ser2448), 13 each for p-mTOR (Ser2481) and Rictor, 5 each for p-Rictor, and 6 each for enzymatic activity assays. These subsets were chosen at random from the pool based on quantity of available samples and matched for age and sex. All lanes were equally loaded for total protein (ranging from 30 to 60 μ g) within any given gel. The control group consisted of 7 females and 13 males, while the AD group consisted of 10 females and 10 males. Mean ages were 71.5 ($\pm$ 10.3) for controls and 77.0 ($\pm$ 10.2) for the AD group. Mean PMI was 18.9 h ($\pm$ 6.3) in the controls and 14.7 h ($\pm$ 6.6) in the AD subjects (not significant). Clinical and specimen processing details of these cases are further detailed [59]. Whole brain samples from AD-like double transgenic mice (Jax 5864 APPsw/PS1-DeltaE9, mean 12 months of age) were provided by the Ann Romney Center for Neurologic Disease. Lysis buffers used were 1% Tween-20 in TEN (40 mM Tris-HCl; pH 7.5, 1 mM EDTA; pH 8.0, 150 mM NaCl) and TBS (20 mM Tris-HCl and 150 mM NaCl), respectively. Whole brain samples prepared from 3 day-old Long Evans rat pups were given bilateral intracerebral (i.c.) injections of streptozotocin (STZ), a model of central insulin resistance and sporadic AD. Control rats were given i.c. saline [60].

Cell culture and antibodies

SH-SY5Y, N2a, and C2C12 cell lines (ATCC, Manassas, VA; SIGMA-ALDRICH, St. Louis, MO) were grown in DMEM supplemented with 10 or 20% fetal bovine serum (FBS;

Invitrogen, Carlsbad, CA) and maintained below 60% confluence for passaging. Cultures of C2C12 at or above 90% confluence were differentiated into myotubes in DMEM, 2% adult horse serum (differentiation medium). Antibodies used include: anti-mTOR (Cell Signaling, recognizing C1 and C2), p-mTOR (Ser2448, Ser2481), p-4EBP1, phospho-AMP-activated protein kinase α (p-AMPK, Thr172), p-Rictor (Thr1135), p-Akt (Ser473, Thr308), neuron-specific enolase (NSE, SantaCruz), Akt, Rictor (Bethyl Laboratories), and 6E10 (Covance).

Primary neuron cultures

PCNs were cultured from E18 Sprague-Dawley rat fetal cortex (Charles River, Wilmington, MA) as described [61, 62]. Briefly, isolated fetal cerebral cortex was dissociated into single cells and then seeded into 6-well plates coated with poly-D-lysine at 1×10^6 cells per well. PCNs were cultured in neurobasal medium (Invitrogen, Carlsbad, CA) containing 2% B27 without insulin, 25 mM D-glucose, 0.5 mM L-glutamine, and 1% penicillin/streptomycin for 7 days before experiments.

Infection of SY5Y, N2a, PCN, and C2C12 myotubes with Adenovirus (Adv) or Herpes Virus (HSV) and extract preparation

The Adv Tet-On and TRE-A β virus system was used to control expression of signal-peptide-A β_{42} leading to accumulation of monomers and oligomers in various cellular compartments (vesicles, cytosol, mitochondria) [63, 64]. SY5Y, N2a, and PCNs were infected with Adv-A β /TetOn (4:1 ratio) for 24 h before doxycycline induction (2 μ g/ml) for an additional 24–18 h. C2C12 were infected on day 3 following switch to differentiation medium. Wild type Rictor and a constitutive active, non phosphorylatable mutant 1135A were generously provided by Dr. Rachael Neve and M.S. Mazei-Robinson [65]. HSV-LacZ served as control infection. Multiplicity of infection was 1.0. Cell extracts were prepared in Lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM Na₃VO₄, 1% Tween20, 10% glycerol, 1 mM Na₄P₂O₇, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin A, 1 μ g/ml aprotinin, 0.1 mM PMSF, and protease inhibitor cocktail; Roche) and were stored at -80°C until used.

Western blot analysis

Whole cell extracts were used directly for western blot analysis (30–60 μ g). Extracts from human autopsy and rodent and cultured cells, prepared in lysis buffer, were diluted into Laemmli sample buffer, heated at 95°C for 10 min, cleared by centrifugation, separated on SDS-PAGE, and transferred to PVDF membrane (Immobilon-P; Millipore). Membranes were blocked in TBS, 0.1% Tween-20, and 5% nonfat dry milk. After incubation with primary antibodies (18 h at 4°C in buffer containing 0.1% Tween-20 and 5% nonfat dry milk or 5% BSA and 0.05% NaN₃), blots were washed and incubated in HRP-conjugated secondary antibodies (1:2000 dilution; Cell Signaling). Signals were detected using ECL reagents and resulting signals quantified and graphed using Image J and GraphPad Prism. Protein signals were normalized to NSE or Actin with same results.

mTORC1 and C2 activities

Immunoprecipitations (IPs) of ~ 200 μ g of crude protein extract, same for control and AD patient or mouse brain, commenced with an overnight incubation at 4°C using 2–3 μ g of

primary antibodies (e.g., anti-total mTOR or anti-Rictor). Protein A/G-Agarose was added for an additional 1.5 h. IPs were harvested at $4000 \times g$ for 1 min at 4°C and washed two times in TBS. For kinase reactions, the samples were washed two more times with kinase assay buffer. To determine mTORC1 activity; following IP of total mTOR, we used the specific mTORC1 substrate, p70S6K-GST fusion protein (K-LISA™ mTOR Activity (Calbiochem), according to the suggested protocol). To assay mTORC2 activity; following IP of the specific mTORC2 complex protein Rictor, we used the mTORC2 substrate, inactive recombinant Akt-GST fusion (K-LISA™ Akt Activity (Calbiochem). As positive control for Akt phosphorylation, we used recombinant PDK. Briefly, each assay is ELISA-based in a total reaction volume of 100 µl. After pelleting the beads, 50 µl reaction supernatant is transferred to a glutathione-coated 96-well plate onto which the substrates are immobilized. Substrate phosphorylations were detected using respective phospho-specific antibodies and HRP antibody conjugate followed by TMB development. Spectrophotometric absorbance was recorded at 450 nm, against a reference 595 nm. The specificity of mTOR and Rictor immunoprecipitations and control phosphorylations of their respective substrates were confirmed using western blotting (Fig. 4A).

In vivo-in vitro Rictor assay and Rictor association with the ribosome

SY5Y cell extracts infected with Adv Aβ/TetOn with/without Dox were prepared in lysis buffer. IPs performed as mentioned above using antibodies versus Rictor, PDK, and ribosomal protein L26 (RPL26). Kinase reactions took place in kinase buffer (25 mM Tris, pH 7.5, 2 mM DTT, 0.1 mM Na₃VO₄, 10 mM MgCl₂, and 200 µM ATP) using wt Akt recombinant protein as substrate (see above). Western blots were developed using anti p-Akt (Ser473). In another measure of mTORC2 (Rictor) function, endogenous RPL26 was first immunoprecipitated. The association of the ribosome protein with endogenous Rictor was then checked on western blot using anti-Rictor [66].

Cell viability

SH-SY5Y cells were washed twice in warm DPBS and incubated in 1 ml DMEM containing 0.5 mg (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT; Molecular Probes, Eugene, OR) for 2–3 h at 37°C and 5% CO₂. The medium was aspirated and the cells were washed twice with pre-warmed DPBS. The formazan salts were dissolved in 1 ml pure ethanol before use. Cells were homogenized by repetitive pipetting and centrifuged for 5 min at 4500 rpm, and the supernatant collected. Absorbance was read against an ethanol blank at 590 nm.

³H-leucine incorporation assay

Protein synthesis was determined using the methods of Freed et al. [67]. Briefly, SY5Y cells in 24-well plates with/without induction of Aβ were stimulated with serum for 48–72 h in the presence of 2 µCi/ml [³H]leucine. The culture medium was aspirated, cells rinsed twice with phosphate-buffered saline, and then harvested at room temperature using isopropanol containing 0.1 N HCl. The lysate was transferred to scintillation vials and beta emission determined in 5 ml Ecolume scintillation fluid (ICN Pharmaceuticals) using a LS6500 scintillation counter (Beckman Coulter, Fullerton, CA).

Autophagy detection

Autophagy is a natural, destructive, and recycling mechanism that disassembles, through a regulated process, unnecessary or dysfunctional cellular components [68]. Autophagy detection was followed using the procedure provided in the Cyto-ID Autophagy detection assay (Enzo Life Science). SY5Y cells with/without expression of A β and with rapamycin (1 μ M) were treated with Cyto-ID Green Autophagy detection reagent and DAPI stain. The cells were fixed in 4% PEA solution for 15 min and analyzed by fluorescence microscopy.

In vitro p-Akt detections and activity levels

PDK, Rictor, and Akt1 were immunoprecipitated overnight from 100 μ g of extract from SY5Y cells using a 1:100 dilution of antibodies. The following morning, 40 μ l of 50% slurry of protein G-Agarose (PGA) (Roche) was added for 1.5 h. The collected beads were washed twice in buffer [1X PBS, 0.5% NP-40, 0.1 mM Na₃VO₄] and twice in kinase buffer [25 mM Tris (pH 7.5), 2 mM DTT, 0.1 mM Na₃VO₄, 10 mM MgCl₂, 200 μ M ATP]. A β peptides, prepared as ADDLs [69] and characterized [59], were added (10 μ M). Finally, GSK-3 fusion protein (1 μ g/50 μ l) was added in the presence of kinase buffer and the reaction (50 μ l) incubated for 30 min at 30°C. The reaction was stopped by adding 40 μ l of Laemmli buffer. 15 μ l of sample was loaded onto a 10% polyacrylamide gel.

RESULTS

Human and animal model brain studies

From a set of 20 AD and 20 age-matched control autopsy cases (Table 1), a maximum of 19 and a minimum of 5 independent patient-brain samples were fractionated. The corresponding densitometric readings showed that phospho-mTOR (Ser2481, Ser2448), corrected for total mTOR, were upregulated, similar to several previous investigator findings. However, a decrease in total mTOR (relative to control protein, NSE) appears to account for most of this change in ratio compared to normal subjects (see Fig. 1 charts). Phospho-mTOR levels were not appreciably different compared to control. Similarly, total Rictor (mTORC2) levels were reduced in AD brain. Notably, phospho-Rictor (Thr1135) levels corrected for NSE were significantly lower in AD. Thus, there was no net change in p-Rictor / total Rictor ratio (Fig. 1, upper chart row). Autophagy marker LC3A/B and mTOR substrate phospho-4EBP1 levels were unchanged in human AD samples compared to control (quantification not shown).

Similar immunoblots of brain samples from two rodent AD models, emulating genetic (APPsw/PS1-DeltaE9 transgenic) and metabolic (STZ rat) drivers of AD pathogenesis are depicted in Fig. 2A and B. In the transgenic model, we found a tendency to higher phospho-mTOR (Ser2448, Ser2481) levels (ratio corrected for total mTOR), in the transgenic samples compared to their control littermates but not reaching significance (lower chart row). Also similar to the AD cases, there was a slight trend toward lower Rictor and total mTOR protein levels in these samples. As in the human AD cases, previous, no differences in p-Rictor or p-4EBP1 ratios corrected for cognate total proteins were noted (Fig. 2A).

Several of the aforementioned findings were confirmed in the STZ rat brain samples in Fig. 2B. For instance, phospho-mTOR (Ser2448 and Ser2481), corrected for total levels, were increased in the rats given intracerebral STZ compared to their sham injected controls (middle chart row). Again, loss of total mTOR (relative to NSE) was mostly responsible for this appearance of activation by ratio. Phospho-Rictor levels trended toward a decrease (upper chart row). Interestingly, eIF-4E-binding protein 1 (4EBP1) and p-4EBP1 levels were significantly upregulated and activated relative to control in this model (bottom chart row). These results, including the change in 4EBP1 levels in the STZ rat model but not in APPsw/PS1 deltaE9 mice or human brain, suggested possible differences in the absolute activity state of mTOR and Rictor kinases.

This led us to directly assay their enzymatic function to phosphorylate cognate substrates, p70S6K-GST fusion protein and inactive recombinant Akt peptide respectively, using an *in vivo-in vitro* approach. As quantified in Fig. 3, both mTORC1 and C2 (Rictor) activities were significantly depressed in human AD (top row), APPsw/PS1deltaE9 mice (middle row) and in IC STZ-treated rat (excepting the Rictor activity downtrend, $p = 0.08$) brain samples compared to their controls. As control, both activities in this assay were found sensitive to rapamycin (Fig. 4A). The result is opposite that of one group [45], whereas another found evidence in APP(sw)/PS1deltaE9 mice for a decrease in mTOR activity accompanied by an expected increase in autophagy [49]. The Fig. 3 results are also in line with reports of downregulation of mTOR signaling in various cellular and animal models of AD [48, 52, 53].

mTORC1 and C2 activity in an amyloid expressing neuronal cell line

To test these mTOR findings in a neuronal cell model of amyloid expression causing insulin signaling changes, mitochondrial derangements, and toxicity, we induced A β ₄₂ expression with doxycycline (Dox) in SH-SY5Y cells infected with an Adenovirus construct [59, 64, 70]. As reported in this model, Akt activation by insulin is inhibited, resulting in a relative state of insulin resistance (Supplementary Figure 1, bottom, 20 nM insulin). In lysates from such cells that express A β , but not in the absence of Dox, mTORC1 activity is significantly reduced. On the other hand, mTORC2 (Rictor) activity remained unaffected by A β expression (Fig. 4A and B, left). In a control experiment, mTORC1 activity appeared more sensitive to rapamycin than did mTORC2 (Rictor) (30 and 15% inhibitions, basal state; 20 and 10% inhibitions, insulin-stimulated conditions, respectively) (data not shown). Treatment with 20 nM insulin in the last 30 min before harvest only partially overcame the rapamycin effects over mTORC1 and C2. Interestingly, addition of A β expression to the rapamycin treatment synergistically inhibited mTORC2 (Rictor) but not mTORC1 activity under either basal or insulin conditions (data not shown). The A β -rapamycin interaction effects are further explored below, but these results suggest that an A β -induced state of insulin resistance also involves mTORC1 activity downstream of Akt. In the western example provided for these *in vivo-in vitro* based activity assays (Fig. 4B, below), the function of immunoprecipitated (IP) Rictor to phosphorylate Akt is shown to be insensitive to A β whereas IP phosphoinositide dependent kinase-1 (PDK-1) activity to phosphorylate Akt is inhibited. To probe another Rictor signaling step, its activation by association with the ribosome [66], we immunoprecipitated ribosomal protein Rpl26 (60S subunit) from cells

expressing A β . The physical association between Rictor and Rpl26 in Rpl26 pull downs was modestly reduced by A β (Fig. 4C, upper). In this case, insulin is shown to both increase their physical association (as expected, Fig. 4C lower panel) and easily overcome the A β effect (Fig. 4C quantification, right).

Neuronal survival, protein synthesis, and autophagy

We next tested the roles of mTOR in viability, protein synthesis, and autophagy in cells under stress from A β expression. mTORC1 in particular has the important role to regulate autophagy, an essential neuroprotective process employed by cells to dispose misfolded proteins and damaged organelles in AD [24, 49, 55, 57]. In Fig. 5A, SH-SY5Y cells show an over 50% reduction in MTT reduction under Dox (24 h) conditions (bar 2 versus 1). However, in the co-presence of rapamycin (0.5 μ M; 24 h), there was a surprisingly significant reversal of toxicity, indicating that even the lowered residual mTOR activity is detrimental to the cell under A β pressure. High dose insulin addition was equally cytoprotective. A β expression inhibits cellular mTOR activity in the aforementioned coupled *in vivo-in vitro* assay. Therefore, we predicted that mTOR's function to engage protein synthesis and downregulate autophagy would be similarly affected. In Fig. 5B, A β expression (Adv+Dox, bar 4) significantly impaired 3H-leucine incorporation under both basal and serum-added conditions. Cyclohexamide control is shown below. In Fig. 5C and quantified below, either A β expression (+Dox) or rapamycin treatments markedly increase autophagic vesicle buildup. In Fig. 6 western panel (row 8 down and plotted to right), A β expression significantly increased autophagy markers LC3A/B. These results are consistent with autophagy activation [71].

mTOR signaling changes in A β ₄₂-expressing cell lines under insulin conditions

We used the AdvTetOn A β ₄₂-Dox induction construct in SY5Y, C2C12 myotube, and N2a cells to examine changes to mTOR-related signal molecules by western blot in Fig. 6 and Supplementary Figure 2. A β expression did not affect phosphorylation of mTOR (Ser2448, Ser2481) or Rictor. Normalized phospho-/total mTOR and /total Rictor and /NSE ratios are quantified in Fig. 6, right. There was no change in phospho substrate 4EBP1, except after rapamycin was added (Supplementary Figure 2). As previously published, p-Akt/tot Akt was inhibited by A β expression (6E10 blot shown in last panel). Interestingly, the mTOR negative regulator AMPK was stimulated (phosphorylated) in A β -expressing cells, which could partially account for deactivation of mTOR activity in Fig. 4A. PhosphoS792-Raptor and phospho-p70S6K levels also remained unchanged in our A β ₄₂ expression model, whereas the expected decreases occurred after rapamycin treatment (results not shown). Control lanes with rapamycin also produced the expected declines in phospho-mTOR, phospho-raptor (S792, not shown), and even Rictor (see also [15]), but the co-expression of A β did not further change this. These results were largely replicated in two other cell lines (Supplementary Figure 2). Curiously, while Rapa by itself had little effect on basal pAkt, it significantly reversed the inhibition of Akt phosphorylation by A β under insulin conditions in both SY5Y cells (Fig. 6) and myotubes (Supplementary Figure 2), consistent with the Fig. 5A cell viability result.

The sum of a large body of literature examining changes to both Akt and mTOR in various AD models suggests the direction of activation may be in either direction depending on the complexities of cell lineage or genetic background, metabolic state, timing, age variables and other contexts. For our model, we determined Akt and Rictor phosphorylations at various time points after doxycycline induction and found that phospho-Akt S473 levels remained consistently inhibited (SY5Y cells, Supplementary Figure 3). Using a different vector to encode intracellular A β (HSV) in N2a cells, we found the same inhibition of insulin (20 nM)-stimulated Akt S473 phosphorylation compared to control HSV (encoding peptide FLT) (not shown). However, the pT308-Akt signal was diphasic indicating a possible compensatory normalization by day 2, only to fall further on subsequent time points (Supplementary Figure 3). Here too, phospho-Rictor (T1135) corrected for total Rictor protein remained unchanged over time (as in Fig. 6 at 2 days), with the exception in cultures that were lysed as far out as 6 days. When Akt activity was tested over time using p-GSK as a substrate, a similar diphasic response, as with T308, was obtained (Supplementary Figure 3).

Rictor expression reverses A β ₄₂ induced signaling changes and toxicity

The above data suggests that while cellular Rictor activity and phospho levels may be impervious to intracellular amyloid peptide, it is nonetheless deficient in total levels and activity in AD and animal models for reasons other than A β accumulation. Since one of its primary roles is to facilitate Akt activation, we reasoned that overexpression might be beneficial to relieve insulin resistance. We designed a dual viral expression experiment in primary rat cortical neuron cultures to test the hypothesis that Rictor expression will reverse the A β -induced insulin resistance. Cell cultures were first exposed to AdvA β and induced with Dox as above. Then in the last 6 h, Rictor was overexpressed through infection with HSV-Rictor constructs before harvest. Rictor, both wt and the constitutive, non-phosphorable 1135A mutant reversed both the p-Akt S473/T308 defect and A β -induced toxicity relative to control HSV encoding LacZ (Fig. 7). The accompanying immunocytochemistry confirmed Rictor expression.

Rictor reverses in vitro effects of amyloid oligomers on Akt

Lastly, we used a complete *in vitro* assay system to determine both Akt activation (phosphorylation) and activity status [59] in the presence of purified Rictor protein. Synthetic A β peptide was prepared as ADDLs (see method). Immunoprecipitations of Rictor, PDK, and Akt proteins from SY5Y cells were incubated with a GSK fusion protein substrate. In Fig. 8 (upper gels and graph), Rictor addition not only stimulated phosphorylation of purified Akt (lane and bar 4 versus 2) but completely overcame the ADDL inhibitory effect (lane and bar 5 versus 3). In Fig. 8 (lower gels and graph), phospho-GSK peptide levels are reduced in reactions containing ADDLs (lane and bar 3 versus 2). Rictor addition reverses the decline, even surpassing control levels (lane and bar 6 versus 3 and 2). Control Actin additions did not affect basal activation (lane and bar 5 versus 2) or ADDL inhibitions (lane and bar 7 versus 3).

DISCUSSION

There have been conflicting reports in the literature on the direction of mTOR signaling changes in AD brain and in mouse models. The reported changes in phospho-protein levels are often not complemented with enzymatic activity data. Moreover, investigation into mTORC2 has been underreported. For instance, in western blot studies of frontal brain from AD, Down syndrome, mild cognitive impairment, and preclinical AD patients, increased ratios of p-Akt (Ser473), p-PI3K (Tyr508), p-mTOR (Ser2448), and p-p70S6K (Thr389) over their respective total protein levels were found by one group, interpreted as evidence for the overactivation of this signaling axis. Correlations with decreased autophagy marker expression and increased inhibitory phosphorylation of IRS-1 were also reported [10, 11]. Similar abnormal activation markers have been found by at least two other groups [34, 37, 41, 72]. Although the molecular cause behind the autonomous activation of PI3K/Akt (and consequently of mTOR downstream of that) in AD brain has not been explained, the collateral resistance to insulin/IGF action [73] has been clearly linked to the feedback inhibitory phosphorylation of IRS-1 [73, 74].

In recent studies, levels of phospho and total mTOR signaling parameters revealed no differences in human AD [47] or were downregulated in cellular and animal models of AD [48, 52, 53]. Furthermore, APP(sw)/PS1deltaE9 mice displayed increased autophagy markers, again accompanied by decreased mTOR activity [49]. The role of systemic insulin resistance (T2DM) in modifying mTOR signaling in AD was recently probed using rat models of T2DM (STZ IP and high fat diet) and AD (hippocampal A β injections). Compared with the Control, T2DM and AD groups, total mTOR protein and mRNA levels in hippocampus, as well as the phosphorylation of tau protein, were significantly increased only in the combined T2DM+AD group, but not between the control and experimental AD group [75]. Finally, mTORC2 (Rictor) levels were only examined in one study of AD and found to be unchanged but not further explored [38].

We examined mTOR variables, with attention to mTORC2 (Rictor), in AD brain samples and in two mouse models. Then we made comparisons with results from cell- and *in vitro*-based experiments where A β is either expressed or peptide added, thereby isolating its effect from other AD-relevant processes and gene products. We found in a set AD frontal cortex samples that both total and p-Rictor (T1135) levels were depressed. Although the corresponding ratio of phospho/total Rictor was not significantly changed (but trending down), the activity level was depressed by ~1/3. Interestingly, total mTOR levels were also depressed, but phospho-mTOR remained unchanged, resulting in an increase in the apparent ratio resembling activation. Activity assays, however, showed basal inhibition of mTOR in the AD condition. In a double transgenic AD model (APPsw/PS1), neither Rictor or mTOR protein activation status (p-S2448mTOR, p-4EBP1) were changed relative to control littermates. Yet Rictor and mTOR enzymatic activities were depressed as well, similar to AD brain. In a mouse model of central insulin deficiency (STZ IC) producing well characterized AD changes [60,76], we found a combination of depressed Rictor levels and an increase in phospho/total mTOR ratio, similar to AD brain. Moreover, phospho and total 4EBP1 levels and their ratio were elevated, suggesting mTOR activation as in previous studies. However, all activity levels were depressed in this model too. These data emphasize that it is important

to complement phospho levels of these kinases and their substrates, a proxy for activity, in brain tissue studies with enzymatic assays.

Moving to a cell culture model (N2A, SH-SY5Y, or C2 myotubes) and employing adenoviral-directed expression of A β ₄₂, mTOR pathway components were not significantly changed (p-mTOR, p-Rictor, total mTOR, and Rictor, p-4EBP1; Fig. 6), more in keeping with the transgenic than the STZ model data. Yet when mTOR and Rictor activities were assayed, A β expression resulted in mTOR inhibition whereas Rictor activity was resistant to it (Fig. 4AB). As expected, Rictor activity was also more resistant to rapamycin than mTOR. Moreover, the activity of Rictor to phosphorylate Akt *in vitro* proved resistant to A β expression *in vivo*. This property to overcome A β -induced insulin resistance is an important point discussed again below. In the same experiments, A β expression increased the activating AMPK T172 phosphorylation. This is another interesting result because AMPK, itself a nutrient sensor and cell energy broker, serves as a powerful negative regulator of mTOR. It plays an important role in cellular energy homeostasis under activating conditions of low substrate and ATP levels [77, 78]. AMPK inhibits mTORC1 via raptor or TSC phosphorylations [79, 80] and supports IRS-1 and Akt expressions. When AMPK is inhibited, for instance in the palmitate model of insulin resistance, mTOR is stimulated [81]. The observed stimulation of AMPK by A β , perhaps compensatory, may partially explain the reduction in mTOR activity observed here [82].

We further tested the inhibition of mTOR activity in A β cultures by examining downstream effects on autophagy and protein synthesis. Downregulation of mTOR signaling facilitates autophagy [83] whereas mTOR positively regulates protein synthesis by phosphorylating p70S6K and 4EBP1 [84]. Consistent with a decline in mTOR activity, LC3A/B levels were increased. This change correlates with the observed increase in autophagy vesicles. Also consistent in this framework, we found that protein synthesis was depressed compared to control condition.

Whether mTOR inhibition in our AD and cell models is detrimental (neurotoxic) or an incomplete protective or compensatory response is inconclusive. To begin testing this we examined what further inhibition with rapamycin might do in the A β expression cell model (see Fig. 6; p-mTOR, Rictor results) and found that the additional mTOR removal was cytoprotective (Fig. 5A). Recent results using palmitate-induced inflammation and insulin resistance also support that mTOR induction, aided by AMPK inhibition, or even residual mTOR activity under certain conditions, are detrimental to the cell [81, 85, 86]. For instance, rapamycin enhances cell viability under endoplasmic reticulum stress and apoptotic conditions, consistent with the pro-apoptotic, anti-autophagic role of mTOR under nutrient rich conditions [87–89].

Importantly, under homeostatic conditions mTOR activation by growth factors (e.g., insulin, BDNF) via PI3K/Akt is vital to the cell [90]. In neurons for instance, mTOR supports size, growth, synaptic plasticity, and dendritic spine numbers [1]. In skeletal muscle, knockout of either Raptor or mTOR or rapamycin treatment results in a muscular dystrophy [91, 92]. Moreover, rapamycin can be toxic to mitochondrial respiration and biogenesis via peroxisome proliferator-activated receptor gamma coactivator (PGC-1 α) disruption [5, 6,

21] and can produce insulin resistance, including inhibition of Akt phosphorylation, IRS-2 levels, and glucose uptake [93]. However, under pathological conditions, mTOR status becomes complicated with often conflicting results. For instance, in AD brain and under experimental AD conditions, pathologic hyperactivation of mTOR (pS2481) and p70S6K is tied to increased tau translation, phosphorylation, and relocalization [33, 34, 94, 95]. This may in turn be related to the basal hyperactivation of Akt reported in particulate (but not cytosolic) fractions in early and mid (but not late) stage AD [37, 74]. It is suggested that A β directly inactivates PTEN and disinhibits PI3K [11, 42]. A β has separately been implicated in directly activating mTOR using transgenic models [44, 45, 96].

How these events happen mechanistically is unknown and untested. The increase in inactivating phosphoS9 of GSK3 β (Tau kinase 1) in these reports does not explain how tau is overphosphorylated in AD. Some studies in fact show basal or stimulated mTOR and Akt are deactivated or unchanged in various transgenic and experimental AD models [48, 50–52, 97]. Our finding that rapamycin is beneficial (regardless of mTOR basal activation status) is consistent with results that mTOR inhibition ameliorates cognitive deficits and pathology in AD mice [44,46] and disease manifestations and proteotoxicity in other neurodegenerative models [97, 98].

The issue of insulin resistance and amyloidosis in AD brain has been extensively reviewed [99–101]. The most cited mechanism is inhibitory phosphorylation at insulin receptor substrate 1 (IRS-1) S616, 636 and decrease in IRS levels [74, 102]. However, this was only convincingly demonstrated just recently in postmortem AD brain [73]. Other mechanisms include reduced numbers and activity of insulin and IGF-1 receptors [74, 101]. Whereas Akt and mTOR were also found to be activated under basal conditions and correlated with oligomeric A β levels, it was the inhibited PI3k/Akt signaling response to insulin stimulation that was most impressive (90% reduction) and perhaps more relevant [73]. As pointed out, the targeting of basal IRS-1 phosphorylation may actually involve kinases other than mTOR [73]. Our results also stress the importance of downregulated Akt phosphorylation by insulin in cells induced to express A β (insulin resistance), over changes to the basal state of Akt activation. We also point to the PDK-Akt activation sequence as a target in this form of insulin resistance and to the potential of mTORC2 stimulation to overcome it. A recent proteomics study of neural cells expressing wt mTOR, in fact found that upregulated mTORC2, but not C1, increased cell viability by facilitating pro-survival and suppressing caspase-mediated apoptotic genes, as well as by stimulating p-Akt (Ser473/Thr308) [103]. This and our result are consistent with mTORC2 survival promoting functions [104, 105].

After finding that mTORC1 inhibition with rapamycin stimulates autophagy (Fig. 5C), partially corrects Akt phosphorylation (Fig. 6) and relieves A β toxicity (Fig. 5A), we next tested if stimulation of mTORC2/Rictor will do the same. Overexpression of Rictor was cytoprotective and restored p-Akt in Fig. 7. Moreover, in an *in vitro* paradigm, the addition of Rictor protein completely reversed A β oligomer-induced inhibition of p-Akt and Akt activity to phosphorylate GSK3 β (lane 6 versus 3, Fig. 8).

With respect to the divided literature on the direction of basal Akt or mTOR activation markers among the various models, it is relevant to consider both disease severity and

chronicity. In our forced viral expression paradigm, both p-Akt and activity to phosphorylate GSK appeared biphasic, suggesting activation early and inhibition at later times (Supplementary Figure 2). Several models also reflect such compensatory and exhaustion signaling phases [97, 106, 107]. We acknowledge that our direct A β ₄₂ expression model may not fully reproduce the pathophysiology of A β metabolism as when derived from mutant APP constructs in cells or in transgenic animals. Nevertheless, in previous work, we showed that viral vector-based A β expression had the same subcellular fractionation into microsomes and mitochondria [108] and co-localization within endoplasmic reticulum and multivesicular bodies as reported in the transgenic 2576 mouse (APP^{swed}) [62]. Further, the same Akt signaling change was found comparing the one model to the other [64]. In the current experiments, our double transgenic animal and A β -expression cell model also share having no major changes to either phospho-mTOR or phospho-Rictor levels and inhibited mTORC1 activity. However, the vectorized-A β approach did not affect Rictor-C2 activity whereas in the animals it did. Although one explanation could be based on an A β expression versus APP processing difference, we favor other major differences in these models for the result and the conclusion that mTORC2 is resistant to intracellular A β ₄₂. Finally, our viral-based cellular model yielded the same results with respect to inhibited Akt activation parameters as *in vitro* experiments using synthetic A β (Figs. 6, 7, and 8 and Supplementary Figure 2).

Our primary aim is not to conclude the basal activation status of mTOR in AD, but rather to demonstrate that stimulation of mTORC2 could be beneficial to overcome insulin resistance in AD. It could even complement a rapamycin-based strategy. The balance between IRS inhibition (mTORC1 negative feedback) and Akt responsiveness to insulin (mTORC2/Rictor positive feedback) could be swung in favor of homeostatic insulin signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Rictor and mTOR levels in control and Alzheimer's disease (AD) brain. Representative immunoblot (upper), relative quantifications (below). Human brain samples (Braak stages V-VI) were extracted in lysis buffer and immunoblotted. An equal amount of total protein (30~60 μ g) was loaded per well across all lanes in a given gel. Soluble A β signals developed with antibody 6E10 are indicated in the upper panel, lowest row. The number of patients tested is given in each of the lower figure graphs ($n = 5$ to 19). Each patient sample in turn was fractionated as a replicate from 2–3 times. Total Rictor, phospho-Rictor (Thr1135), and total mTOR levels normalized to NSE were significantly decreased in AD samples

compared to the control. Accordingly, the phospho-mTOR (Ser2481, Ser2448) / total mTOR ratios were higher in these AD samples and the p-Rictor/total Rictor ration was unchanged relative to control. LC3 A/B and p-4EBP1 levels showed no significant change relative to control. A β ₄₂ (6E10) signals were quantified as RDU (relative densitometric units). Mean and sd: control patients 10843 $\pm$ 2825; AD patients 22062 $\pm$ 11568, p value = 0.0024; n =13 each. Bottom graphs: * p < 0.05, ** p < 0.01, *** p < 0.001 (two-tailed Student's t test); error bars are s.e.m.

A

Fig. 2.

A) Double transgenic mice (APP^{sw}/PS1-DeltaE9, mean 12 months of age) brain samples were extracted in TBS and immunoblotted as in Fig. 1A. Total Rictor and mTOR levels trended downward whereas p-mTOR corrected for total trended upward (non-significant). Phospho-Rictor and p-4EBP1 levels were not different between transgenic and littermate control animals. The graphs summarize quantitations of densitometric measurements made from each protein signal ($n = 6$ animals each group, replicate data over two independent westerns, error bars are s.e.m.).

B) mTOR, Rictor, and 4EBP1 protein levels from rats given intracerebral STZ. Frozen brain samples were extracted in TBS and immunoblotted as above. Decreased levels of total mTOR and Rictor proteins with respect to NSE (not significant) are reflected in elevated ratios of phospho-mTOR/total. In this model, mTORC1 substrate p-4EBP1 levels were elevated compared to control, conveying that mTOR activity may be increased (however, see Fig. 3). The graphs represent quantifications of each protein signal corrected for either NSE or total cognate protein: * $p < 0.05$, ** $p < 0.01$ (two-tailed Student's t test), $n = 6$ STZ, 6 sham animals, extracts ran in duplicate, error bars are s.e.m.).

Fig. 3.

Immunoprecipitated mTOR and Rictor proteins from Alzheimer's disease (AD) patient, double transgenic, and STZ-treated brain samples were incubated with cognate substrates, p70S6K-GST fusion protein, and wt recAkt, respectively. See methods for commercial-based mTOR and Akt activity assays. mTOR and Rictor activities were both decreased in all three AD models relative to their controls (excepting non-significance in the Rictor-STZ samples). * $p < 0.05$, ** $p < 0.01$ (two-tailed Student's t test), n = individual patient samples or animals numbers tested, error bars are s.e.m.

Fig. 4.

mTOR (A) and Rictor (B) activities were determined in lysates from SH-SY5Y cells infected with Adenovirus Aβ₁₋₄₂/TetOn (coupled in *vivo-in vitro* assay). Aβ expression was induced by the introduction of doxycycline (Dox) 2 μg/ml over 24–36 h.

Immunoprecipitations (IPs) of mTOR and Rictor from 100 μg of cell extract protein were incubated with substrates p70S6K-GST fusion protein or recombinant Akt-GST, respectively (ELISA assay, as in Fig. 3). mTOR, but not Rictor, activity was sensitive to Aβ expression ($n = 3$). In the single control experiments shown right, the effect of rapamycin alone and together with Aβ expression (Dox) on mTOR (A) and Rictor (B) activity is

probed. Both rapamycin and insulin treatments were applied in the last 30 min before harvest. Western blots are shown at the far right which confirms IPs of the two kinases and their substrate phosphorylations as used in the ELISAs. In the western blot along the bottom, IPs of Rictor and PDK from lysates of cells uninduced (–) and Dox-induced (+) for A β expression were incubated with wt recAkt (unstimulated substrate) in the presence of ATP and kinase buffer. Rictor activity was not affected by A β expression, as above, whereas PDK activity to stimulate Akt phosphorylation was inhibited. C) Rictor binding to ribosome protein RPL26 is affected by A β expression. IP of RPL26 and western blot of Rictor from cells exposed to AdvA β +/- Dox and with (+) or without (–) insulin treatment. The graphical quantification to the right shows modest sensitivity to A β expression, easily reversed by the insulin treatment. Bottom straight- and IP-western control blot showing insulin stimulates Rictor binding to RPL26. $n = 2$, * $p < 0.05$ compared with control, + $p < 0.05$ compared with A β (two-tailed Student's t test, error bars are s.e.m.).

Fig. 5.

A) Cell viability assessment using the MTT reduction assay. The reacted formazan salts were dissolved in pure ethanol and measured photometrically. Intracellular Aβ expression (Adv Aβ+Dox) provoked cell death (bar 2, $p < 0.01$). Partial reversal by rapamycin (1 μM, bar 3, $p < 0.05$) or insulin (100 nM, bar 4). B) 3H-Leucine incorporation levels in the presence of Aβ₄₂. SY5Y cells with/without Dox induction in the presence of 2 μCi/ml [³H]leucine. Cells were maintained under basal or serum stimulation (20%, 72 h) conditions. Protein synthesis was arrested by intracellular Aβ expression. Serum treatment only partially reversed Aβ toxicity (+, bar 8 versus 4). Cycloheximide (CHX, 10 μM) control experiment given below. C) Autophagic vesicles were counted in SY5Y cells with/without expression of Aβ (+/-Dox) or with rapamycin (1 μM control). Cells were developed using Cyto-ID Green Autophagy detection reagent that co-localizes with LC3B (not shown) and Dapi. Rapamycin

or A β are shown to increase autophagic vesicle counts. $n = 4$ experiments, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (two-tailed Student's t test, error bars are s.e.m.).

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Fig. 6.

mTOR and Rictor signal changes. SY5Y cells were infected with AdvA β ₄₂/TetOn virus and A β expression induced during 24~36 h of Dox (2 μ g/ml). Cultures were co-treated with rapamycin (R) or vehicle (C) and insulin for the last 30 min before harvest. A β expression did not change phosphorylations of mTOR or Rictor but did reduce phospho-Akt levels (plotted on figure right). Similar results obtained examining Akt substrate, p-CREB (not shown). Glucose deficient (5 mM) and in excess (50 mM) conditions did not substantially change results (not shown). Total proteins, mTOR, Rictor, Akt1, and NSE were unchanged. Phospho-AMPK and LC3A/B-II levels were increased by A β . See Supplementary Figure 2 for similar C2C12 and N2A results. * p < 0.05, + p < 0.05 (two-tailed Student's t test, n = 3, error bars are s.e.m).

Fig. 7. Rictor expression rescues primary cortical neuron cultures from Aβ expression-inhibited Akt phosphorylation and cell death. PCNs from rat embryo were infected with the AdvAβ+Dox inducible expression system (48 h). Rictor overexpression was achieved by co-infection with non-inducible HSV encoding CA Rictor 1135A mutant or wt in the last 6 h before the harvest. HSV induced LacZ expression served as inert control for Rictor. Quantification of the western ($n = 3$), WST cell viability ($n = 6$), and Rictor TMB-HRP photometric ($n = 6$) developments are shown figure right. Correction of p-Akt for total and pT308-Akt gave same results. Phospho-Rictor levels underwent similar changes (not shown). * $p < 0.05$, + $p < 0.05$.

0.05 (two-tailed Student's *t* test). Lower panel is immunohistochemistry of HSV infections on fixed cells; Bright field (BF), DAPI nuclear stain (middle) and anti Rictor (red color).

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Fig. 8.
In vitro reactions emulating cell-based conditions. Unstimulated wt PDK-1, Rictor, Akt, and Actin proteins were purified from 90% confluent SY5Y cell extracts. The mixture contained kinase buffer, GSK fusion protein (crosstide), ATP and PIP3. A β was added in the form of synthetic ADDLs. Rictor additions restored the inhibition of the PDK to Akt activation sequence by A β , as shown by an increase in both variables, p-Akt (upper, lane 5) and p-GSK (lower, lane 6) westerns, respectively. Addition of purified Actin yielded activity levels equivalent to control (Lane, bar 5 versus 2 and 7 versus 2). * $p < 0.05$, + $p < 0.05$, ++ $p < 0.01$ (two-tailed Student's t test) ($n = 3$, error bars are s.e.m).

Alzheimer's disease and control brain demographics. Four sample sets of same number AD and Control brain samples as shown were received from the McLean Brain Bank and pooled (20 AD, 20 Control). From this were taken representative subsets of age- and sex-matched samples for Figs. 1 and 3 data

Table 1

	Pathological diagnosis	Stage	Age	Sex	Postmortem interval (h)	Tissue used
Control		Normal	60	F	17.6	BA10, Temporal pole
Control		Normal	58	F	31.7	BA10, Temporal pole
Control		Normal	58	M	17.5	BA10, Temporal pole
AD		Braak V	75	F	10.6	BA10, Temporal pole
AD		Braak V	92	M	7.1	BA10, Temporal pole
AD		Braak V	92	F	5.0	BA10, Temporal pole
Control		Normal	82	M	24.1	BA9, Temporal pole
Control		Normal	52	F	17.9	BA9, Temporal pole
Control		Normal	78	M	25.3	BA9, Temporal pole
AD		Braak VI	83	F	14.3	BA9, Temporal pole
AD		Braak VI	87	F	16.9	BA9, Temporal pole
AD		Braak VI	62	M	25.3	BA9, Temporal pole
Control		Normal	61	F	10	Prefrontal cortex
Control		Normal	81	M	13	Prefrontal cortex
Control		Normal	75	M	9	Prefrontal cortex
AD		B&B III	73	M	11	Prefrontal cortex
AD		B&B V/VI	82	F	12	Prefrontal cortex
AD		B&B V/VI	85	F	11	Prefrontal cortex
Control		Normal	67	M	23.5	Temporal Pole
Control		Normal	73	M	24.0	Temporal Pole
Control		Normal	76	M	12.3	Temporal Pole
Control		Normal	77	F	20.6	Temporal Pole
Control		Normal	68	M	26.5	Temporal Pole
AD		Braak VI	64	M	24.0	Temporal Pole
AD		Braak VI	67	F	20.7	Temporal Pole
AD		Braak VI	67	M	14.5	Temporal Pole
AD		Braak VI	91	M	22.5	Temporal Pole

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Pathological diagnosis	Stage	Age	Sex	Postmortem interval (h)	Tissue used
AD	Braak VI	66	M	10.5	Temporal Pole
Control	Normal	66	M	17.8	BA 9
Control	Normal	66	M	24.6	BA 10
Control	Normal	85	M	20.8	BA 10
Control	Normal	89	F	14.1	BA 10
Control	Normal	83	F	8.9	BA 9
Control	Normal	74	M	18.6	BA 10
AD	Braak VI	74	F	21.8	BA 9
AD	Braak VI	77	F	14.7	BA 9
AD	Braak VI	71	F	7.8	BA 9
AD	Braak VI	90	M	6.6	BA 9
AD	Braak VI	78	M	22.5	BA 9
AD	Braak VI	61	M	22.8	BA 9