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mTOR and Neuronal Cell Cycle Re-entry: How Impaired Brain Insulin Signaling Promotes Alzheimer's Disease

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Abstract

A major obstacle to pre-symptomatic diagnosis and disease-modifying therapy for Alzheimer's disease (AD) is inadequate understanding of molecular mechanisms of AD pathogenesis. For example, impaired brain insulin signaling is an AD hallmark, but whether and how it might contribute to the synaptic dysfunction and neuron death that underlie memory and cognitive impairment has been mysterious. Neuron death in AD is often caused by cell cycle re-entry (CCR) mediated by amyloid- β oligomers (A β Os) and tau, the precursors of plaques and tangles. We now report that CCR results from A β O-induced activation of the protein kinase complex, mTORC1, at the plasma membrane and mTORC1-dependent tau phosphorylation, and that CCR can be prevented by insulin-stimulated activation of lysosomal mTORC1. A β Os were also shown previously to reduce neuronal insulin signaling. Our data therefore indicate that the decreased insulin signaling provoked by A β Os unleashes their toxic potential to cause neuronal CCR, and by extension, neuron death.

Keywords

Amyloid- β oligomers; tau; Rac1; Alzheimer's disease; cell cycle re-entry; insulin; diabetes

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1. Introduction

The most conspicuous histopathological features of Alzheimer's disease (AD) brain include two types of poorly soluble aggregates: extracellular plaques made from amyloid- β ($A\beta$) peptides and intraneuronal tangles assembled from the neuron-specific protein, tau. Complementing plaques and tangles as criteria for diagnosing AD are behavioral symptoms that result from synaptic dysfunction of neurons that mediate memory and cognition, and the death of those neurons [1]. Although soluble forms of $A\beta$ and tau work coordinately to drive AD pathogenesis [2], a detailed mechanistic understanding of the normal signaling networks that go awry in AD and the pathological networks that are activated has remained an elusive goal.

Despite the limited degree to which signaling processes in AD are understood, several important clues have been recognized. One such clue concerns impaired brain insulin signaling. Glucose uptake within the brain is reduced in AD patients [3], and post-mortem studies have revealed dramatically reduced expression of receptors for insulin and insulin-like growth factor 1 in the hippocampus and hypothalamus [4]. Although type 1 and type 2 diabetes are strong risk factors for AD, decreased insulin signaling in AD brain typically occurs even in patients without systemic diabetes, prompting the suggestion that AD represents brain-specific, or type 3, diabetes [3, 4]. It is widely assumed that diminished insulin signaling must elicit broad deleterious effects on brain metabolism, but exactly how those effects lead to synaptic dysfunction and neuron death has been puzzling.

Neuron death in brain regions affected in AD can be massive, involving loss of up to 2/3 of hippocampal CA1 neurons [5] and 90% of the neurons in selective neocortical areas [6]. The majority of these post-mitotic neurons apparently die by an ironic pathway: ectopic cell cycle re-entry (CCR) [7, 8]. Although differentiated neurons in normal adult brain are permanently post-mitotic and thereby arrested in G0, up to 10% of the neurons in regions affected by AD show signs of re-entering the cell cycle at any given time during pre-symptomatic disease stages. These cells apparently never divide, however, and their eventual disappearance from brain is matched by a commensurate loss of neurons in the same regions [9]. AD thus represents a striking contrast to cancer in the sense that AD symptoms arise in part because cells that attempt to divide die instead, whereas cancer symptoms result from cell proliferation run amok. It follows naturally that identifying proteins required for neuronal CCR and defining the relevant signaling networks hold promise for diagnosing AD at its earliest stages and developing new therapies to impede AD progression.

We recently reported the framework of a signaling network that causes CCR in AD [10]. The mechanism involves $A\beta$ oligomer ($A\beta$ O) induced activation in parallel of three protein kinases - fyn, protein kinase A (PKA) and calcium/calmodulin-activated kinase II (CaMKII) - which then must respectively phosphorylate tau at Y18, S409 and S416. All of this occurs in cultured neurons within hours of initial $A\beta$ O exposure, and thus may be a seminal process in AD pathogenesis. We also demonstrated tau-dependent CCR *in vivo* by comparison of AD model mice that either expressed or lacked tau genes, and confirmed prior reports [10-12] that mTOR kinase activity is required for neuronal CCR.

mTOR is a constituent of two multi-protein complexes, mTORC1 and mTORC2, that regulate fundamental cellular behavior, such as protein synthesis, cell growth, cell cycle progression and autophagy, in response to cell surface receptors that detect insulin, growth factors and nutrients, like amino acids. These functions require tight regulation of mTOR kinase activity at lysosomes and the plasma membrane (PM) [13, 14], and mTORC1 and mTORC2 cross-regulate each other by positive and negative feedback mechanisms [15, 16]. mTOR kinase activity is chronically elevated in AD brain [17], and overexpression or suppression of mTOR in AD model mice respectively aggravate or relieve AD-like pathology and behavioral deficits [18, 19]. We now provide evidence for how mTOR is dysregulated by A β Os and works through tau to drive insulin-sensitive CCR, a prelude to neuron death in AD.

2. Methods

2.1 A β Os

Lyophilized, synthetic A β ₁₋₄₂ (GL Biochem, Ltd.) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (Sigma-Aldrich) to ~1 mM and evaporated overnight at room temperature. The dried powder was resuspended for 5 minutes at room temperature in 40-50 μ l dimethylsulfoxide to ~1 mM and sonicated for 10 minutes in a water bath. To prepare oligomers, the dissolved, monomeric peptide was diluted to ~400 μ l (100 μ M final concentration) in Neurobasal medium (Life Technologies/GIBCO), incubated 24-48 hours at 4° C with rocking, and then centrifuged at 14,000 g for 15 minutes to remove fibrils. For all experiments with primary neurons (see next section), A β Os were diluted into tissue culture medium to a final concentration of 1.5 μ M total A β ₁₋₄₂.

2.2 Primary neurons

Primary neuron cultures were prepared from dissected brain cortices of E17/18 wild type (WT) or tau knockout (KO) [20] mice as described previously, and maintained in Neurobasal medium supplemented with B27 (Life Technologies/GIBCO) [10]. Lentivirus transductions were performed at 7 days in culture, and all experiments were completed after neurons had been in culture for a total of 10-11 days. For CCR and most mTOR kinase assays, neurons were grown in B27-free Neurobasal for 5 hours before addition of A β Os. To identify neurons in S-phase, bromodeoxyuridine (BrdU; Sigma-Aldrich) was added to medium to 10 μ M simultaneously with A β Os. For mTOR kinase and immunofluorescence assays shown in Figure 2A, 5A and 5B, neurons were incubated in Hank's balanced salt solution (HBSS) for 2 hours prior to a 30 minute exposure to A β Os, 1 μ M insulin, or a mixture of 0.398 mM L-arginine plus 0.8 mM L-leucine. For Figure 5C and Supplemental Figure S1, neurons were incubated in B27-free Neurobasal for 2 hours before addition of insulin (to 1 μ M) or amino acids (0.398 mM L-arginine plus 0.8 mM L-leucine), 10 minutes after which A β Os were added. The cells were fixed and stained for immunofluorescence or In-Cell western blot analysis [10] 24 hours later. Rapamycin, NSC23766 (both from Calbiochem) and bovine pancreatic insulin (Sigma-Aldrich, cat. no. I5500) were diluted into medium to 100 nM, 10 μ M and 500 nM, respectively. Cultures were preincubated with Torin1 (500 nM; kindly provided by Dr. John Lazo, University of Virginia) 2 hours before addition of A β Os. Cell-permeable TAT peptides were purchased from GL Biochem, Ltd.

2.3 Antibodies

See Table S1.

2.4 cDNA Constructs and shRNA Sequences

The pEGFP-Rac1_{WT} vector was a gift from Drs. Martin Schwartz (Yale) and Konstadinos Moissoglu (NIH). The pEGFP Rac1_{C178S} construct was provided by Dr. Miguel del Pozo (Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III, Spain). These constructs were amplified by PCR and transferred to the lentiviral vector pBOB-NepX (Addgene plasmid 12340; deposited by Dr. Inder Verma, The Salk Institute) between the BamHI and HpaI sites using the following primers: forward 5' TGC GGA TCC GCA ATG GTG AGC AAG GGC GAG GAG 3' and reverse: 5' CGC GTT AAC GCG TTA CAA CAG CAG GCA TTT TCT 3'. The WT human 2N4R tau cDNA was described previously [10]. The human 4EBP1 WT and double-phospho null T37A/T46A were provided by Dr. Jing Zhang (Johns Hopkins). These constructs were amplified by PCR and cloned into the lentiviral expression vector, pLJM1 FLAG Raptor-H-Ras25 (see below for vector information), between the SalI and EcoRV restriction sites by using the following primers: forward 5' TGC GTC GAC TAT GAT ATG TCC GGG GGC A 3' and reverse 5' TGG CTA TAG AAT GTC CAT CTC AAA CTG 3'. An S262A human 2N4R tau vector was provided by Dr. Alejandra Alonso (College of Staten Island). This construct was amplified by PCR and transferred to pBOB-NepX between the AgeI and HpaI sites using the following primers: forward 5' TTA ACC GGT ATG GCT GAG CCC CGC CAG 3' and reverse 5' ATT GTT AAC CTA CAA ACC CTG CTT GGC CAG 3'. The pLJM1 FLAG-Raptor-Rheb15 and pLJM1 FLAG-Raptor-H-Ras25 lentiviral expression constructs were from Addgene (plasmids 19312 and 26637, respectively; both were deposited by Dr. David Sabatini, Massachusetts Institute of Technology).

For shRNA knockdowns, the following published oligonucleotide targeting sequences were inserted into the pLKO.1 vector (Addgene plasmid 10878; deposited by Dr. David Root, Broad Institute) following the manufacturer recommendations:

1. shNCAM = 5' CGA CTT CTT TGG CCA CTA TAC3'.
2. shRac1 = 5' G AGG AAG AGA AAA TGC CTG 3'.
3. shRala = 5' A AGG CAG GTT TCT GTA GAA 3'.
4. shGαs = 5' A GGC GCA GCG CGA GGC CAA 3'.
5. shTsc2 = 5' GGT GAA GAG AGC CGT ATC 3'. Knockdown efficiencies were monitored by western blotting.
6. Plasmids to knock down Raptor and Rictor were purchased from Addgene (plasmids 21339 and 21341, respectively; both deposited by Dr. David Sabatini). Knockdown efficiencies were monitored by western blotting for 4EBP1/2_{pT37/pT46}.
7. Plasmid to knock down Nprl3 was from The RNAi Consortium of the Broad Institute (TRCN0000175195). Knockdown efficiency was monitored by western blotting.

8. The scrambled shRNA plasmid was from Addgene (Plasmid 1864; deposited by Dr. David Sabatini).

2.5 Lentivirus Production and Infection

pBOB-NepX or pLKO.1 expression plasmids, and the packaging vectors, pSPAX2 and pMD2.G (Addgene plasmids 12260 and 12259, respectively; both deposited by Dr. Didier Trono, École Polytechnique Fédérale de Lausanne) were transfected using Lipofectamine 2000 (Life Technologies) into HEK293T cells grown in 15 cm Petri dishes to ~80% confluence in Dulbecco's MEM (Invitrogen) supplemented with 10% HyClone cosmic calf serum (GE Healthcare). Each transfection was with 30 µg total DNA at a 50%/37.5%/12.5% ratio of expression vector/pSPAX2/pMD2.G. Transfection medium was replaced with fresh medium after 24 hours, and lentivirus-conditioned medium was collected 48 and 72 hours after the start of transfection. Lentiviral particles were concentrated in a Beckman Coulter Optima LE-80K ultracentrifuge for 2 hrs at 23,000 rpm (95,000 g_{av}) at 4° C in an SW28 rotor, resuspended in 400 µl Neurobasal medium and stored at -80° C in 20 µl aliquots. Cultured neurons were infected with a viral MOI of 5 and incubated for 24 hours at 37° C, after which lentiviral medium was replaced with fresh Neurobasal plus B27.

2.6 Microscopy

Cultured neurons growing on #1 thickness, 12 mm round glass coverslips in 24-well dishes were rinsed in PBS and fixed for 15 minutes in 3.7% formaldehyde. Next, they were washed and permeabilized in washing buffer (0.2% Triton X-100 in PBS) 3 times for 5 minutes each, incubated for 60 minutes in blocking buffer (1 M glycine, 0.2% Triton X-100, 3% BSA in PBS) and transferred to PBS.

For experiments in which cell surface binding of AβOs was analyzed, all steps described in the prior paragraph were done in the absence of detergents. Binding of AβOs to neurons was substantial at 10 days in culture and did not significantly increase by 15 days in culture (Supplemental Figure S1).

For BrdU uptake experiments, nuclear BrdU antigenicity was enhanced prior to antibody labeling by incubating neurons in the following order: 1) 10 minutes in 1 N HCl on ice ; 2) 10 minutes at room temperature; 3) 20 minutes at 37° C in 2 N HCl; 4) acid neutralization with 0.1 M sodium borate pH 8.6 for 12 minutes at room temperature; and 5) transfer to PBS. Fixed neurons were incubated for 1 hour each with primary and secondary antibodies diluted into PBS containing 3% BSA, with several PBS washes after each antibody step. Finally, coverslips were mounted onto glass slides using Fluoromount-G (Southern Biotech, Inc.). Samples were imaged on a Zeiss Axiovert 100 [10]; or a Nikon Eclipse Ti equipped with a Yokogawa CSU-X1 spinning disk head, 60× and 100× 1.4 NA Plan Apo objectives, and 405 nm, 488 nm, 561 nm and 640 nm lasers. Colocalization of mTOR or TSC2 with LAMP1 was quantified by the Manders Coefficient plug-in (http://www.uhnresearch.ca/facilities/wcif/software/Plugins/Manders_Coefficients.html) for ImageJ (<http://rsbweb.nih.gov/ij/index.html>), which is based on the Pearson Correlation Coefficient [21].

Floating 50 µm thick mouse brain sections were obtained from 3×Tg [22], Tg2576 [23] and Tg2576 mice hemizygous for mTOR expression in brain [24]. 3×Tg mice were fed mouse

chow containing microencapsulated rapamycin from 2-18 months of age [19]. Sections were rinsed in PBS for 5 minutes, then incubated with blocking buffer (PBS plus 5% normal goat serum) for 2 hours at room temperature. Sections were incubated in suspension with primary antibodies for 16 hours at 4° C and with secondary antibodies for 2 hours at room temperature. Antibodies were diluted into PBS containing 2% normal goat serum and 0.05% Tween 20 (PBS/Tween20), and incubated with tissue sections with gentle rocking. Three 10 minute PBS/Tween20 washes followed each antibody step. After the final wash the sections were rinsed with PBS, and mounted between #1 thickness coverslips and glass slides using Fluoromount G. Sections were imaged on the Nikon Eclipse Ti, or on a Leica TCS SP5 X scanning laser confocal microscope equipped with a 63×, 1.4 NA Plan Apo objective and a 488 nm argon laser line for imaging AlexaFluor 488 and a white light laser tuned to 590 nm for imaging AlexaFluor 594. For statistical analysis, at least 2000 NeuN-positive cells were counted per brain section along the cortices.

Human cortical brain biopsy samples, routinely obtained during placement of ventricular peritoneal shunt, were from 12 white, non-Hispanic patients who presented clinically with normal pressure hydrocephalus (Supplemental Figure S7). Samples were fixed within 30 minutes of the procedure in 10% zinc formalin. All biopsy samples were archival material that remained after diagnosis, and the Institutional Review Board of the University of Virginia approved the use of these tissues for the procedures described here. Following fixation, the samples were embedded in paraffin, sectioned to 4 µm thickness and mounted on glass slides. Paraffin was removed using xylenes before further processing for immunofluorescence, which was performed as described above for mouse brain sections, except that the tissue was adsorbed to glass for all steps and primary antibody incubations were for 2 hours at room temperature. Immediately prior to sealing the slides to glass coverslips with Fluoromount G, lipofuscin autofluorescence was eliminated using the Autofluorescence Eliminator reagent (Millipore) following the manufacturer's protocol. Immunoperoxidase labeling was performed using the Dako Envision Flex detection reagent on a Dako Autostainer platform.

2.7 Immunoblotting

Samples were resolved by SDS-PAGE using 10 or 12% acrylamide/bis-acrylamide gels and transferred to 0.22 µm nitrocellulose (Bio-Rad). Membranes were blocked with Odyssey blocking buffer (LI-COR), and were incubated with primary antibodies and secondary IRDye-labeled antibodies (Tables S2 and S3) diluted into antibody buffer (Odyssey blocking buffer diluted 1:1 in PBS/0.2% Tween 20). All antibody incubations were for 1 hour at room temperature or overnight at 4° C, and 3 washes of 5 minutes each with PBS/0.1% Tween 20 were performed after each antibody step. Membranes were dried between sheets of filter paper prior to quantitative imaging with an Odyssey imaging station (LI-COR).

2.8 In-Cell Western Blots

Primary neurons were cultured for 10 days as described earlier, but were plated into 12-well dishes at a density of 250,000 cells per well. Neurons were incubated in B27-free Neurobasal for 2 hours before addition of insulin (to 1 µM) or amino acids (to 0.398 mM L-arginine plus 0.8 mM L-leucine), 10 minutes after which AβOs were added. 24 hours later

cells were fixed in 4% PFA for 15 minutes and then washed with PBS without detergents for 15 minutes (3×, 5min. each). Fixed cells were incubated at room temperature for 60 minutes with Odyssey Blocking Buffer (LI-COR), then for 1 hour with primary (6E10 mouse anti-A β and rabbit anti-NrCAM) and infrared-labeled IRDye secondary antibodies (LI-COR) diluted into Odyssey Blocking Buffer. Following each antibody step, the cultures were washed 5 times in PBS. The dishes were allowed to dry at least for 2 hours in the dark, and then were scanned using the LI-COR Odyssey imaging station according to the manufacturer's instructions, with a 3.0 mm offset and a scan intensity of 3.0. The intensity ratio for 6E10 to NrCAM was calculated and normalized to the level in untreated samples, and was expressed as mean $\pm$ s.e.m.

2.9 Lipid Raft Isolation

Cultured neurons ($\sim 1 \times 10^6$ cells growing in a 10 cm petri dish) were homogenized in ice-cold TNE buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EGTA) containing 0.1% Triton X-100 (Sigma-Aldrich). Each homogenate (1 ml) was adjusted to 40% sucrose by the addition of 1 ml of 80% sucrose in TNE and placed at the bottom of a 5 ml ultracentrifuge tube. A 5–30% discontinuous sucrose gradient (0.5 ml of 5% sucrose and 2.5 ml of 30% sucrose, both in TNE) was layered on top of the homogenate, after which the sample was spun in a Beckman Coulter TLX tabletop ultracentrifuge using a TLS-55 swinging bucket rotor at 52,000 rpm (180,000 g_{av}). 12 fractions of 410 μ l each were collected from the top of the centrifuge tube. Lipid raft proteins partitioned into the lowest density fractions.

3.0 Immunoprecipitation

WT cortical neurons were transduced with lentiviruses to express either GFP-Rac1 WT or GFP-Rac1_{c178S} for 72 hours. Cells were then lysed in ice cold CHAPS buffer (120 mM NaCl, 1 mM EDTA, 40 mM HEPES pH 7.4, 50 mM NaF, 10 mM β -glycerophosphate, 0.3% CHAPS, 1 mM Na₃V0₄, 1 mM PMSF, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1 mM MgCl₂) for 30 minutes at 4° C. Cellular debris was cleared by centrifugation at 17,000 g for 10 min at 4° C in a bench top centrifuge. The supernatant was collected and incubated on a vertical rotator with 5 μ g/ml of either anti-GFP or anti-NeuN for 2 hours at 4° C. Finally, 25 μ l of protein G magnetic beads (New England Biolabs) was added to each supernatant and incubated for 1 hour at 4° C. Immunoprecipitates were washed 3 times with CHAPS buffer and eluted by adding SDSPAGE sample buffer. For competitive assays with cell permeant TAT peptides, WT cortical neurons expressing GFP-Rac1_{WT} were treated with 20 μ M TAT peptides for 5-6 hours, and then were processed for immunoprecipitations as just described.

3.10 Statistics

The student t-test was used for analyzing bar graphs, the tables shown in Figures 4C and 6B, and the line graph shown in Supplemental Figure S5D. A minimum of 150 cells per condition from a total of at least 3 experiments for all bar graphs and from 2 experiments for the tables was used. Data from 3 experiments were used for the line graph.

4. Results

4.1 mTORC1 and mTORC2 are required for neuronal CCR

To determine whether one or both mTOR complexes are required for CCR we treated cultured wild type (WT) mouse cortical neurons with shRNA to reduce the levels of Raptor or Rictor, which are respective defining subunits of mTORC1 and mTORC2. To measure knockdown efficiency, we used quantitative western blotting to monitor phosphorylation of the mTORC1 substrate, 4EBP1/2 (at T37 and T46), and the mTORC2 substrate, Akt (at S473). To monitor CCR simultaneously, we exposed A β O-treated and control untreated neurons to bromodeoxyuridine (BrdU), a synthetic nucleotide that can covalently incorporate into DNA, and used anti-BrdU immunofluorescence microscopy to identify nuclei that had incorporated BrdU and thus had entered S-phase of the cell cycle. The cultures were also stained for immunofluorescence with antibodies to the neuron-specific proteins, MAP2 and tau, to discriminate neurons from the glial cells that were also present.

As shown in Figure 1A, ~5% of the neurons were BrdU-positive in the absence of A β Os, but the level rose to ~40% after A β O treatment. Knockdown of either Raptor or Rictor completely blocked A β O-induced BrdU incorporation by neurons. Similar results for BrdU incorporation were obtained by directly inhibiting mTORC1 with rapamycin, or both mTORC1 and mTORC2 with Torin1 (Supplemental Figure S2). Torin1 also strongly reduced A β O-induced phosphorylation of 4EBP1/2 at T37 and T46, and of another mTORC1 substrate, p70/S6 kinase (S6K) at T389 (Figure 1B). These collective results indicate that both mTORC1 and mTORC2 are required for A β O-stimulated neuronal CCR.

Expression of the nuclear G1 marker, cyclin D1, at a level sufficient for cell proliferation is enabled by the translation regulator, eIF4E, whose activity is inhibited by its binding to 4EBPs [25]. When 4EBPs are phosphorylated at T37 and T46 by mTORC1, however, they dissociate from eIF4E, thereby enabling cell-cycle progression [25]. We found that Tg2576 AD model mice [23] with hemizygous, as compared to homozygous mTOR expression in brain [24], had an ~4 fold reduction in cortical neurons expressing cyclin D1 or the G1/S/G2 marker, Ki67 [26] (Figure 1C). mTOR haploinsufficiency in Tg2576 mice rescues memory deficits [24] and dramatically reduces cortical eIF4E immunostaining (Figures 1C), suggesting that aberrant regulation of translation through the mTOR/eIF4E pathway promotes AD.

4.2 A β Os activate mTORC1 at the PM, but not at lysosomes

mTOR is thought to act primarily at lysosomes and the PM [13, 14, 27, 28]. To determine where A β Os activate mTORC1 in cultured neurons, we forced mTORC1 to accumulate preferentially on lysosomes or the PM by lentiviral expression in cultured neurons of FLAG-Raptor fused either to a lysosome-targeting region of Rheb (FLAG-Raptor-Rheb15) or a PM-targeting region in H-Ras25 (FLAG-Raptor-H-Ras25) [29]. Quantitative western blotting indicated that mTORC1 kinase activity, as measured by 4EBP1/2_{pT37/pT46}/4EBP1_{total}, increased ~2-fold after exposure of neurons expressing FLAG-Raptor-H-Ras25 to A β Os, insulin or both. In neurons expressing FLAG-Raptor-Rheb15, mTORC1 activity rose similarly in response to insulin or insulin plus A β Os, but not

significantly after exposure to A β Os alone (Figure 2A). A β Os also induced nuclear cyclin D1 accumulation in ~25% of both non-transduced neurons and neurons expressing FLAG-Raptor-H-Ras25. In contrast, nuclear cyclin D1 was not induced by A β Os in neurons expressing FLAG-Raptor-Rheb15 (Figure 2B). Insulin thus efficiently stimulates mTORC1 at the PM and lysosomes, while A β Os stimulate mTORC1 only at the PM.

To seek further support for these observations, we also forced 4EBP1 to accumulate at the PM by expressing either FLAG-4EBP1_{WT} or the non-phosphorylatable FLAG-4EBP1_{T37A/T46A} mutant fused to the same PM targeting region of H-Ras-25. A β Os induced nuclear accumulation of cyclin D1 in ~25% of control, non-transduced neurons or neurons expressing FLAG-4EBP1_{WT}-H-Ras25, but less than 15% of neurons expressing FLAG-4EBP1_{T37A/T46A}-H-Ras25 were positive for nuclear cyclin D1 after A β O exposure (Figure 2C). A β O-stimulated phosphorylation of 4EBP1 at T37 and T46 by PM-associated mTORC1 therefore promotes neuronal CCR.

To determine if the PM-specific activation of mTORC1 by A β Os requires tau, we targeted mTORC1 to the PM or lysosomes by respective lentiviral expression of FLAG-Raptor-H-Ras25 or FLAG-Raptor-Rheb15 in primary neurons derived from tau knockout mice (tau KO neurons). In the absence of lentiviral expression, A β Os stimulated 4EBP1/2 phosphorylation only half as well in tau KO neurons as in WT neurons (Supplemental Figure S3B). When FLAG-Raptor-H-Ras25 or FLAG-Raptor-Rheb15 was expressed in tau KO neurons, 4EBP1/2 phosphorylation rose slightly after A β O exposure, but the increases were not statistically significant (Supplemental Figure S3C). These results indicate that the PM-specific activation of mTORC1 by A β Os occurs by a mechanism that requires tau.

4.3 Rac1 targets mTORC1 to PM lipid rafts for CCR

Because we previously found that CCR is not blocked by inhibitors of GSK3 β [10], which negatively regulates mTORC1 [28], or inhibitors of insulin/IGF-1 receptors, which are upstream of mTORC2 [28], we sought alternative mechanisms linking CCR to mTOR. An shRNA knockdown screen of WT cortical neurons revealed that NCAM, G α s and the GTPases, RalA and Rac1, are required for A β O-induced CCR (Figure 3). NSC23766, which inhibits Rac1 by blocking its interaction with guanine nucleotide exchange factors and thereby maintains Rac1 in the GDP-bound state [30], was also found to inhibit CCR (Supplemental Figure S4B,C). These results are consistent with prior findings that NCAM is an upstream regulator of fyn and CaMKII [31], that G α s regulates PKA through its control of adenylyl cyclase [32], that RalA is required for activation of phospholipase D, which generates the mTOR activator, phosphatidic acid [33], and that Rac1 targets both mTORC1 and mTORC2 to membranes by directly binding mTOR [14]. The results further indicate that GTP-bound Rac1 is required for CCR, despite the prior finding that Rac1-GTP and Rac1-GDP bind equally well to mTOR [14]. Consistent with these conclusions, we found that shRNA knockdown of Rac1 in WT cortical neurons to ~40% of normal level caused an ~60% decrease in 4EBP1/2 phosphorylated by mTORC1 at T37/T46 (Figure 4A), and ~70% drops in A β O-induced BrdU uptake and cyclin D1 expression (Figure 4A and Supplemental Figure S4A).

Binding of Rac1 to mTOR requires the RKR sequence (amino acids 186-188) located near the C-terminus of Rac1 [14]. To test the importance of the Rac1/mTOR interaction for CCR we treated primary neurons with the cell-permeable TAT peptide [34], or TAT modified by addition at its C-terminal of a Rac1 peptide containing the WT RKR sequence (TAT-Rac1_{WT}) or a sequence in which RKR was mutated to AAA (TAT-Rac1_{3A}). Compared to neurons treated with TAT or TAT-Rac1_{3A}, neurons treated with TAT-Rac1_{WT} had an ~50% reduction in baseline levels of S6K_{pT389} and 4EBP1/2_{pT37/pT46} and an ~80% reduction in A β O-induced BrdU uptake (Figure 4B). Because CCR can be blocked by binding of mTOR to the TAT-Rac1_{WT} peptide (Supplemental Figure S5A), we conclude that Rac1 binding to mTOR is necessary for CCR and is competitively inhibited by TAT-Rac1_{WT}.

Membrane binding of Rho GTPases is required for their interaction with downstream effectors and regulators of their catalytic activity. For Rac1, membrane targeting is mediated by a C-terminal, polybasic region and geranylgeranylation of the cysteine in the adjacent CAAX domain [35]. PM targeting of Rac1 also requires its translocation to lipid rafts [36, 37], which are low density membrane microdomains that integrate cellular signaling process [38]. Lipid raft recruitment of Rac1 depends on palmitoylation at C178, and regulates Rac1 activation and function [35]. In WT neurons, partitioning of palmitoylation-deficient GFP-Rac1_{C178S} into lipid rafts is reduced by ~60% compared to GFP-Rac1_{WT} (Supplemental Figure S5B,C). Moreover, cyclin D1-positive nuclei in neurons expressing GFP-Rac1_{WT} rose ~5-fold after A β O exposure, but was not increased by expression of GFP-Rac1_{C178S} (Figure 4C). Conversely, A β O-induced, mTORC1-catalyzed phosphorylation of 4EBP1/2 was significantly reduced by expression of GFP-Rac1_{C178S} compared to GFP-Rac1_{WT} (Supplemental Figure S5D). Finally, immunoprecipitation demonstrated that the palmitoylation-deficiency of GFP-Rac1_{C178S} did not affect its ability to interact with endogenous mTOR (Figure 4D). Altogether, these data imply that excessive, A β O-mediated targeting to lipid rafts of Rac1 and mTORC1 to which it is bound triggers downstream signaling required for CCR.

4.4 Lysosomal mTOR activation by insulin or amino acids blocks A β O-induced CCR

Coordinated positioning of mTORC1 and two of its inhibitors on lysosomes regulates mTORC1 activity at that location. One inhibitor, TSC2, dissociates from lysosomes in response to insulin [13] or amino acid [27] stimulation, while the other inhibitor, the GATOR1 complex, is inactivated by amino acids [39]. mTORC1 activation at lysosomes is thus coupled to rises in insulin or amino acids (Figure 5A).

Because A β O-induced activation of mTORC1 occurs at the PM, but not at lysosomes (Figure 2), we compared the effects of A β Os, insulin, and a mixture of arginine plus leucine on the lysosomal content of mTORC1 and TSC2 in cultured WT cortical neurons. We confirmed by western blotting of 4EBP1/2_{pT37/pT46} that a 30 minute exposure to any of these agents leads to an ~2-fold activation of mTORC1 (Figure 5A). Using immunofluorescence, we also found that insulin or the amino acids causes an ~10% increase in the level of lysosomal mTOR, as judged by its co-localization with the lysosome marker, LAMP1, without altering the level of lysosomal TSC2. In contrast, A β Os did not induce recruitment of mTOR to lysosomes, but did increase lysosomal TSC2 by >30% (Figure 5B).

These findings suggest that the failure of A β Os to activate lysosomal mTORC1 (Figure 2) reflects the lack of resultant mTOR recruitment and gain of TSC2 on lysosomes, neither of which occurs in response to insulin or amino acids. The rise of net 4EBP1/2 phosphorylation induced by A β Os (Figure 5A) can be explained by activation of mTORC1 at the PM (Figure 2).

We next determined the effects of insulin and amino acids on CCR. Neither insulin nor arginine plus leucine could induce neuronal CCR, as judged by their failure to cause nuclear accumulation of cyclin D1. Remarkably, though, either of these agents could prevent A β Os from causing CCR (Figure 5C) without affecting the level of A β O binding to the neuronal surface (Supplemental Figure S1A,B). We also found that shRNA knockdown of either TSC2 or the GATOR1 complex subunit, Npr13 [39] (Supplemental Figure S6), blocked A β O-induced CCR (Figure 5D). CCR thus results when A β Os stimulate activation of mTORC1 at the PM in the absence of concomitant mTORC1 activation at lysosomes.

4.5 mTOR-dependent tau phosphorylation at S262 is required for CCR

Abnormal tau phosphorylation is a hallmark of AD and non-Alzheimer's tauopathies [40]. Tau is commonly phosphorylated at just a few residues per molecule in normal brain, but AD tau is hyperphosphorylated [40] with ~45 phosphorylated residues having been detected [41]. Only in a few cases, though, including neuronal CCR, has site-specific phosphorylation been linked to specifically altered tau function [10, 42-45]. Because A β has been reported to induce tau phosphorylation at S262 by S6K [46], which itself becomes phosphoactivated by mTORC1 [28], we investigated whether tau_{pS262} is required for CCR. First, we determined that Torin1 or shRNA-mediated Rac1 knockdown reduced the level of tau_{pS262} in primary neurons by ~40% or ~75%, respectively (Figure 6A). Next, we found that lentiviral expression in cultured tau knockout neurons [47] of human WT tau (2N4R isoform), but not S262A mutated tau, supports A β O-induced CCR (Figure 6B). Finally, using AD model mice, we observed that pharmacological inhibition of mTORC1 by feeding rapamycin to the 3 $\times$ Tg strain [22] or genetic reduction of mTOR in the Tg2576 strain [23, 24] leads to a dramatic decrease in tau_{pS262} (Figure 6C). Thus, tau phosphorylation at S262 is regulated by mTOR *in vitro* and *in vivo*, probably indirectly through S6K, and is required for CCR (Figure 6D).

4.6 Coincidence of tau phosphorylation and neuronal CCR in human AD brain

The collective evidence from this study and our prior work on CCR [10] demonstrates that A β O-induced tau phosphorylation at a minimum of four sites – Y18, S262, S409 and S416 – is essential for CCR in cultured neurons or mouse models of AD. Here we show a strong correlation of tau phosphorylation with neuronal CCR in human AD brain tissue as well. Rapidly fixed brain biopsy samples from 12 normal pressure hydrocephalus patients ranging in age from 61 to 87 (see Supplemental Figure S7A for patient data summaries) were analyzed by immuno-peroxidase staining for plaques and tangles by a neuropathologist (JM), who concluded that patients 11 and 12 had AD neuropathologic changes according to established criteria (Supplemental Figure S7B). Further analysis by double immunofluorescence revealed a strong correspondence in the AD tissues between neuronal CCR, as marked by co-localization of Ki67, a nuclear marker for cells that G1, S or G2 of

the cell cycle, with the neuron-specific nuclear protein, NeuN, and the presence of tau phosphorylated at S262, S409 and S416 (Supplemental Figure S7C). Although we were not able to detect tau_{pY18} in any of the tissue samples, these results nevertheless reinforce the conclusion that A β O-induced tau phosphorylation at multiple specific sites underlies neuronal CCR in AD.

5. Discussion

5.1 Analysis of results

Despite sustained and heroic efforts on many fronts, the quest to conquer AD has been a frustrating journey that has not yet yielded any disease-modifying therapies. The reasons for this disappointing state of affairs are legitimate topics of debate, but surely include our currently limited understanding at the molecular level of how normal neuronal homeostasis is altered during the earliest stages of AD pathogenesis. Memory and cognitive deficits, the behavioral hallmarks of AD, reflect the dysfunction and loss of synapses among neurons that mediate memory and cognition, and by the death of those neurons. The study presented here sheds light on how neuron death in AD results from impaired brain insulin signaling [3, 4] enabling the apparent re-entry of post-mitotic neurons into the cell cycle [7-9]. One of the most important features of this ectopic neuronal CCR is that it occurs independently of amyloid plaque and neurofibrillary tangle formation by a mechanism that is initiated by A β Os, the precursors of the plaques, and requires soluble forms of tau, the building blocks of the tangles. Moreover, cultured neurons express CCR markers within hours of A β O exposure, suggesting that CCR represents a seminal process in AD pathogenesis, and by extension, a possible diagnostic and therapeutic target for AD.

Two key players in the CCR signaling network are mTOR and Rac1. The results presented here demonstrate that both mTORC1 and mTORC2 are required for CCR, that A β Os activate mTORC1 at the PM, but not at lysosomes, that GTP-bound Rac1 in lipid rafts is necessary for the membrane targeting and activation of mTORC1, and that CCR depends on mTORC1-dependent tau phosphorylation at S262. We also show that A β O-induced CCR can be blocked by insulin or amino acids, either of which activates mTORC1 at both the PM and lysosomes. We therefore propose that CCR results from mTOR signaling dysregulation, whereby A β O-induced, tau-dependent activation of mTORC1 occurs at the PM, but is somehow prevented at lysosomes.

Besides initiating this signaling web, A β Os have also been shown to reduce the insulin responsiveness of neurons by activating TNF α /JNK signaling that leads to phosphorylation of IRS-1 at sites that block insulin signaling [48-50]. Microglia play an important role in this process because they secrete TNF α in response to A β Os [51]. A β Os are thus a two-edged sword that both reduce insulin signaling and enable a devastating downstream consequence, CCR and eventual neuron death. This explains mechanistically why systemic diabetes is a strong AD risk factor and why impaired brain insulin signaling in the absence of systemic diabetes is also associated with AD symptoms [3, 4]. Figure 6C summarizes our current understanding of the CCR signaling network based on the new data presented here, along with relevant, previously published findings [10-12, 48-50].

How might mTOR become activated by A β Os? One possibility is by perturbation of membrane microdomains corresponding to lipid rafts. Extracellular A β Os incorporate into neuronal membranes and are gradually recruited to lipid rafts by a fyn-dependent mechanism [52]. At the cell surface, Rac1 is preferentially localized in lipid rafts [36], and as shown here in Supplemental Figure S5C, a palmitoylation-deficient, GFP-tagged mutant Rac1 that blocks A β O-induced CCR is less efficiently partitioned into lipid rafts than a GFP-tagged WT Rac1 that effectively permits CCR. It is also noteworthy that every one of the protein kinases we have identified as being required for CCR – fyn, PKA, CaMKII and mTOR (Figure 6C) – are partially enriched in lipid rafts [52-55], as are NCAM and G α s [56, 57], which we identify here as being upstream of fyn and CaMKII, and PKA, respectively, in the CCR pathway (Figures 3 and 6C). These collective findings raise the possibility that A β Os perturb lipid raft structure in a manner that causes activation of the kinases, including mTOR, that are required for neuronal CCR.

Although most of the new mTOR data presented here is directed at mTORC1, we did find that inhibition of mTORC2 blocks CCR as effectively as inhibition of mTORC1 (Figure 1A). This is not surprising in light of the finding that mTORC1 and mTORC2 regulate each other by both positive and negative feedback mechanisms [15, 16]. A key feature of this feedback loop is that mTORC1-catalyzed phosphorylation of S6K leads to mTORC2 disassembly and inactivation, which in turn suppresses mTORC1 activity [15]. Perhaps A β Os alter this delicate balance between the two mTOR complexes by somehow bypassing the inhibitory steps of the feedback loop that functionally connect them.

One of the best known effects of mTORC1 activation at lysosomes is suppression of autophagy [58]. Because A β Os activate mTORC1 robustly at the PM, but not at lysosomes (Figure 2), A β O-induced CCR appears to depend on spatially restricted mTORC1 activation that neither reduces nor enhances autophagic activity. This idea must be considered in the context of *in vivo* studies of AD model mice, which have demonstrated beneficial effects of autophagy upregulation by rapamycin, namely a reduction of plaques, tangles and behavioral deficits [18, 19, 59]. The neutral effects of A β Os on autophagy may therefore be deleterious in two ways. First, by failing to activate lysosomal mTORC1 and thereby suppress autophagy, A β Os evidently dysregulate normal mTOR signaling. Secondly, by failing to reduce the basal activity of lysosomal mTORC1 and thereby enhance autophagy beyond normal levels, A β Os do not stimulate cells to rid themselves of toxic, misfolded forms of A β and tau.

Residing at the center of the CCR story is tau, because without tau A β Os cannot induce CCR either in cultured neurons or *in vivo* [10]. Importantly, mTOR activation and neuronal CCR have also been shown to result from human tau expression in a *Drosophila* tauopathy model [12]. Although our initial study of CCR led us to conclude that tau phosphorylation at S262 is not necessary to drive neurons out of G0 [10], that conclusion was mistaken and based on assaying tau_{pS262} at a single, 24 hour time point after exposing cultured neurons to A β Os. We now know that A β Os induce transient tau phosphorylation at S262, which returns to baseline level by 24 hrs (data not shown). Moreover, because expression of WT, but not S262A tau in tau KO neurons restores their ability to re-enter the cell cycle after A β O exposure (Figure 6B), tau phosphorylation at S262, as well as at Y18, S409 and S416 [10],

are unequivocally required for CCR. With the exception of pY18, we found a strong correlation between the presence of those phospho-tau epitopes and CCR neurons in human cortical brain biopsy samples from AD patients (Supplemental Figure S7). The lack of detectable tau_{pY18} in the human tissue could be due to a neuroanatomical limitation. Tau_{pY18} has been detected in the entorhinal cortex and hippocampus of post-mortem AD brain [60], but the rapidly fixed biopsy samples that we examined were limited to superficial cortical areas. Nevertheless, a major challenge for the future will be to identify the molecular mechanisms that phosphorylated tau orchestrates to drive neurons out of G0 and back into the cell cycle.

The pathogenic A β -tau connection that underlies neuronal CCR must be considered in the context of the numerous failed efforts to treat AD in humans by reducing A β levels and our limited knowledge about tau hyperphosphorylation in most mouse models of AD. The disappointing clinical trials targeting A β have been largely directed at mid to late stage AD patients. The events described here, however, represent A β O-induced, tau-dependent changes that occur in neurons within hours after initial A β O exposure and may not require more sustained A β O insults. Furthermore, none of the tau phosphorylation sites that we identified as being essential for CCR represent canonical AD sites, such as the PHF1 (pS396/pS404), AT100 (pS212/pT214) and AT180 (pT231) phospho-epitopes, that are routinely assayed as indicators of AD tau in AD mouse models and human AD patients. It is therefore noteworthy that we found 3 of the 4 tau phosphorylation sites that are necessary for neuronal CCR – pS262, pS409 and pS416 – to be increased in human AD brain (Supplemental Figure S6).

5.2 Clinical implications

How might the new results presented here impact the clinical management of AD? Let us consider diagnosis first. Analysis of cerebrospinal fluid (CSF) as a possible source of AD biomarkers is a promising area of investigation, as a combination of A β _{x-42} and tau_{pT181} in CSF has already been promoted as a tool that can accurately diagnose AD ~5 years before clinical symptoms are evident [61]. As encouraging as these results are, perhaps earlier accurate diagnosis can be achieved by assaying CSF for other potential AD biomarkers either individually or in combination, such as those described here and in our previous work on CCR [10]. Examples of such biomarkers include tau phosphorylated at sites we have identified as being required for CCR (Y18, S262, S409 and S416) and mTOR substrate proteins whose phosphorylation at specific sites are indicative of abnormally high mTOR activity in brain (4EBP1/2_{pT37/pT46} and S6K_{pT389}).

In terms of potential therapies, the kinases that control tau phosphorylation at sites required for CCR, including mTOR, fyn, PKA and CaMKII, represent possible targets. Because all of those kinases are involved in regulating a variety of cellular process in brain and other tissues, however, the risk of adverse side effects caused by targeting them with drugs is substantial. A noteworthy counterpoint to that concern is the successful use of rapamycin to reduce the histopathology and behavioral decline in the 3 $\times$ Tg strain of AD model mice [18]. Rapamycin is used in humans to treat a few types of cancer, but its modest efficacy and side effects have limited the drug's utility [62]. Perhaps one or more structurally and

pharmacologically related “rapalogs” could overcome these deficiencies of rapamycin for treating humans with AD.

One protein that stands out from the crowd as an attractive target for AD therapy is tau. Because tau expression is nearly confined to neurons, any drugs that target tau with high specificity are unlikely to cause complications outside of the brain, with the possible exception of peripheral nerve. In a plaque-bearing AD mouse model, elimination of one tau gene has been shown to relieve learning and memory deficits [63], raising the possibility that reduction of tau, short of its elimination entirely, would have substantial therapeutic value in humans. Tau reduction could be accomplished in principle by antisense nucleotide therapy, provided reagents that effectively cross the brain-blood barrier can be developed. An alternative approach directed at tau could be to use passive or active immunization to deplete toxic forms of tau from brain. Finally, our finding that insulin blocks CCR opens one more possible therapeutic avenue. Unfortunately, the reduced insulin signaling of AD brain [3, 4] precludes the simple administration of insulin as a likely productive strategy for AD treatment. Nevertheless, because the insulin effect on CCR involves its ability to activate lysosomal mTORC1 (Figure 5), the development of an insulin-independent method for activating lysosomal mTORC1 might reduce neuronal CCR and downstream neuron death to benign levels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Research in Context

1. Systematic review: CCR, the re-entry of post-mitotic neurons into the cell cycle, is a prelude to neuron death that occurs in AD, and along with synaptic dysfunction, accounts for the learning and memory deficits associated with the disorder. This study was initiated to unravel the pathogenic signaling network that causes CCR, and by extension, to reveal potential new diagnostic and therapeutic targets for AD.
2. Interpretation: This study indicates that the previously described ability of A β Os to impair neuronal insulin signaling unleashes their toxic potential to cause tau-dependent CCR. This is because CCR depends on A β Os activating mTORC1 at the plasma membrane, but not at lysosomes, where mTORC1 activation by insulin blocks CCR.
3. Future directions: At a mechanistic level, defining biochemical steps in the CCR signaling network that require tau is a major goal. At a translational level, leveraging our findings to improve AD diagnostics and therapeutics is the main objective.

Fig. 1. mTOR Is Required for CCR *In Vitro* and *In Vivo*

(A) Knocking down Raptor or Rictor inhibits phosphorylation of the respective mTORC1 and mTORC2 substrates, 4EBP1/2 at T37 and T46, and Akt at S473, and blocks AβO-induced, nuclear uptake of BrdU. (B) Torin1 inhibits AβO-induced phosphorylation of the mTORC1 substrates, S6K (at T389) and 4EBP1/2. (C) mTOR haploinsufficiency suppresses neuronal CCR. Error bars in all panels represent s.e.m.

Fig. 2. AβOs Activate mTORC1 at the PM, But Not at Lysosomes

Neurons expressing modified mTORC1 subunits targeted to the PM (FLAG-Raptor-H-Ras25) or lysosomes (FLAG-Raptor-Rheb15) were exposed to AβOs or insulin. (A) 4EBP1/2 phosphorylation at T37 and T46 was increased ~2-fold by a 30 minute exposure to AβOs, insulin or a combination of both. (B) CCR, as monitored by nuclear cyclin D1 localization, was induced by a 24 hour exposure to AβOs when mTORC1 was targeted to the PM, but not to lysosomes. (C) AβO-induced CCR, as monitored by nuclear cyclin D1 expression, was supported by overexpressing 4EBP1_{WT}, but not 4EBP1_{T37A/T46A}, at the PM. Error bars represent s.e.m. for 3 separate experiments.

Fig. 3. NCAM, RalA, Gαs and Rac1 Are Required for CCR

(A) Selection of candidates based on evidence from other systems. (B) Knockdown efficiency of targeted candidates. (C and D) AβO-induced CCR, as monitored by nuclear BrdU uptake, is inhibited by knockdown of NCAM, RalA, Gαs or Rac1. Error bars in B and C represent s.e.m.

Fig. 4. mTOR Targeting to Lipid Rafts by Rac1 Is Required for CCR

(A) Rac1 knockdown reduces 4EBP1/2 phosphorylation, nuclear cyclin D1 expression and BrdU uptake in AβO-treated neurons. (B) TAT-Rac1_{WT} peptide inhibits BrdU uptake, and S6K and 4EBP1/2 phosphorylation, and (see Figure S5) Rac1-mTOR interaction in AβO-treated neurons. (C) Expression of GFP-Rac1_{C178S}, partitioning of which to lipid rafts is impaired (Supplemental Figure S5), blocks CCR in AβO-treated neurons. (D) Rac1-mTOR interaction is not dependent on palmitoylation at C178. Error bars in A and B represent s.e.m.

Fig. 5. Insulin or Amino Acids activate mTORC1 at Lysosomes and Block AβO-induced CCR
 (A) AβOs, insulin or amino acids (arginine + leucine) stimulate an ~2-fold increase in 4EBP1/2 phosphorylation in cortical neurons. (B) Neurons starved of insulin and amino acids for 2 hours were treated with AβOs, insulin or arginine + leucine for 30 minutes, and then were labeled by double immunofluorescence for mTOR and LAMP1, or TSC2 and LAMP1. (C) AβO-induced CCR is blocked by insulin, or arginine + leucine. (D) shRNA knockdown of Npr13 or TSC2 blocks AβO-induced CCR. Error bars in all panels represent s.e.m.

Fig. 6. CCR Requires mTOR-Dependent Tau Phosphorylation at S262

(A) Baseline level of tau_{pS262} in primary neurons is reduced by Torin1 or Rac1 knockdown. (B) Expression of WT, but not S262A tau in tau knockout (KO) neurons restores A β O-induced CCR. (C) Pharmacologic inhibition of mTOR (*ad libitum* access to rapamycin-laced chow from 2-18 months of age) or mTOR haploinsufficiency reduces tau_{pS262} in AD model mice. (D) A model of the neuronal CCR pathway in AD. A β Os initiate two parallel, interconnected pathways that together lead to mTOR dysregulation, which is obligatory for ectopic CCR. One pathway leads from G α s and NCAM to activation of fyn, PKA and CaMKII, which then respectively phosphorylate tau at Y18, S409 and S416. The other pathway stimulates translocation of Rac1-mTORC1 complexes to plasma membrane lipid rafts, where mTOR kinase activity is stimulated towards 4EBP1/2 and S6 kinase, and leads to tau phosphorylation at S262. Tau and mTORC1 connect the two pathways.