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NMDA receptor-mediated calcium influx connects amyloid-β oligomers to ectopic neuronal cell cycle re-entry in Alzheimer's disease

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Abstract

Introduction: Alzheimer's disease (AD) symptoms reflect synaptic dysfunction and neuron death. Amyloid-β oligomers (AβOs) induce excess calcium entry into neurons via N-methyl-D-aspartate receptors (NMDARs), contributing to synaptic dysfunction. The study described here tested the hypothesis that AβO-stimulated calcium entry also drives neuronal cell cycle re-entry (CCR), a prelude to neuron death in AD.

Methods: Pharmacologic modulators of calcium entry and gene expression knockdown were used in cultured neurons and AD model mice.

Results: In cultured neurons, AβO-stimulated CCR was blocked by NMDAR antagonists, total calcium chelation with BAPTA-AM, or knockdown of the NMDAR subunit, NR1. NMDAR antagonists also blocked activation of calcium-calmodulin-dependent protein kinase II (CaMKII), and treatment of Tg2576 AD model mice with the NMDAR antagonist, memantine, prevented CCR.

Discussion: This study demonstrates a role for AβO-stimulated calcium influx via NMDAR and CCR in AD, and suggests the use of memantine as a disease-modifying therapy for pre-symptomatic AD.

1. Introduction

Alzheimer's disease (AD) is a devastating neurological disorder characterized by memory loss and cognitive decline. These behavioral symptoms are caused at the cellular level by synaptic dysfunction and loss, and neuron death, and at the molecular level by toxic forms of amyloid-β (Aβ) and tau that work coordinately to damage synapses [1-3] reduce insulin signaling [4], impair axonal transport [5], and kill neurons [6-8]. While poorly soluble, fibrillar forms of Aβ and tau that respectively are found in plaques and tangles are histopathological hallmarks of AD, soluble oligomeric forms of Aβ and tau are now recognized as being far more toxic [9-11]. It follows naturally that efforts to prevent and treat AD will benefit from advances in our still primitive understanding of the pathogenic signaling mechanisms that underlie the breakdown of normal neuronal homeostasis caused by toxic oligomers of Aβ and tau.

As much as 90% of neuron death in AD may follow ectopic re-entry of neurons into the cell cycle [12,13]. Whereas fully differentiated, healthy neurons are permanently post-mitotic, affected neurons in AD and other neurodegenerative disorders often express molecular markers of the G1 and S-phase stages of the cell cycle [14-17]. Instead of dividing, however, these neurons apparently die after a delay of up to hundreds of days following cell cycle re-entry (CCR) [18]. Previous research from our laboratory defined a CCR signaling network in AD, whereby Aβ oligomers (AβOs) induce activation of multiple protein kinases that catalyze site-specific tau phosphorylation, and a positive feedback loop between phospho-tau and the multi-subunit protein kinase complex, mTORC1, that leads to mTORC1 dysregulation and drives neurons from G0 into G1 [19,20].

Besides triggering CCR and neuron death, AβOs cause excitotoxicity by stimulating excess calcium influx into neurons [21-23]. This disruption of normal calcium homeostasis affects numerous signaling pathways, and can damage and destroy synapses, and lead to abrupt neuron death [24]. A major contributor to this pathological process is the N-methyl-D-aspartate receptor (NMDAR), which permits toxic levels of calcium to enter neurons exposed to AβOs [3,25]. Interestingly, one of the few FDA-approved treatments for AD is memantine, which works by blocking excess calcium entry into neurons via NMDAR [26].

The study described here tested the hypothesis that neuronal CCR and excitotoxic calcium influx via NMDAR share a common mechanistic origin initiated by AβOs. Using AβO-treated primary mouse neuron cultures, we show that CCR is prevented by chelating total cellular calcium, by pharmacologically blocking AβO-mediated calcium influx through NMDAR, or by reducing expression of an NMDAR subunit protein. Moreover, we found that neuronal CCR *in vivo* in Tg2576 AD model mice can be blocked by treating the mice prophylactically with memantine. Taken together, these results mechanistically link AβO-induced calcium influx and neuronal CCR. Moreover, they suggest that memantine, which is used as a drug for modestly relieving symptoms in patients with a clinical AD diagnosis, but does not apparently act as a disease-modifying drug in that context, has the potential to forestall disease progression if administered during pre-symptomatic stages of the disease.

2. Methods

2.1. Animals and Usage

All animal usage and protocols were approved by the IACUC of the University of Virginia. Animals were housed in a barrier facility with *ad libitum* access to food and water on a 12-hour light/dark cycle.

2.2. Neuron dissections and cultures

Cortical neuron cultures derived from E17/18 wild type C57/BL6 mouse embryos prepared and maintained as described previously [20]. Cultures were maintained for 16-18 days prior to experimental manipulations.

2.3. Aβ oligomerization and treatment

AβOs were made from lyophilized Aβ(1-42) (Anaspec, AS-20276-5), which first was resuspended in 1,1,1,3,3,3-hexafluoro-2- propanol (HFIP; Sigma, 105228-5G) and incubated for 4 hours at room temperature to yield a solution of monomeric peptide. 20 µl aliquots were stored at -80° C until ready to use. To prepare oligomers, an aliquot was dried in a speed vac for 3 hours, solubilized in 1 mM in dimethyl sulfoxide (Sigma, D2650-5X5ML), placed in a water bath sonicator for 5 minutes, and then supplemented with Neurobasal medium (GIBCO/Life Technologies, 21103-049) to yield a final peptide concentration of 100 µM. The solution was then placed on an orbiting rocker at 4° C for 48 hours, after which it was centrifuged at 14,000 x g in a tabletop microcentrifuge for 15 minutes to pellet large aggregates. AβO solutions were diluted into neuron cultures to yield a final total Aβ concentration of ~2 µM, and the cultures were processed for immunofluorescence microscopy or western blotting 16-18 hours later. Controls demonstrating

that AβOs, but not Aβ monomers or fibrils, cause CCR were described in a previous publication from our lab [19].

2.4. Immunofluorescence microscopy

All steps were performed at room temperature unless indicated otherwise. Primary mouse cortical neurons grown on #1.5 thickness, 12 mm round glass coverslips were rinsed once with ice-cold phosphate buffered saline (PBS), and then were fixed in 4% paraformaldehyde in PBS for 12 minutes. Following fixation, the cells were rinsed 3 times for 5 minutes each with ice cold PBS, permeabilized for 15 minutes with PBS containing 0.2% Triton X-100 (Fisher, 9002-93-1), and rinsed 3 times for 5 minutes each with PBS. Next, the cells were blocked with PBST (PBS supplemented with 2% bovine serum albumin (BSA; Roche, 03116956001); and 0.1% Tween 20 (Fisher, 9005-64-5) for 1 hour, after which they were labeled overnight with primary antibodies followed by secondary antibodies for 1 hour. Antibodies were diluted into 2% BSA in PBS, and after each antibody incubation step the cells were rinsed 3 times for 5 minutes each with PBS. Finally, the coverslips were rinsed with ultrapure water and mounted onto slides using Fluormount-G (Southern Biotech, 0100-01) containing 1% 1,4-diazabicyclo[2.2.2]octane (Sigma, D27802-25MG), an anti-quenching agent.

Brain tissue sections were obtained from mice that were transcardially perfused first with 10 ml of phosphate buffer (PB; 0.1 M sodium phosphate, pH 7.6), followed by 10 ml of 4% paraformaldehyde in PB at a flow rate of 1.5 ml/minute. Next, brains were removed and placed in 4% paraformaldehyde in PB for 24 hours, washed 3 times in PBS, incubated in 30% sucrose overnight, and flash frozen using dry ice. Frozen tissue was stored at -80° C until ready for sectioning to 40 µm thickness on a Microm HM 505 E cryostat.

Sagittal brain sections were placed into wells of a 12-well tissue culture dish, rinsed briefly with PBS, then rinsed 3 times for 15 minutes each in PBS + 0.3% Triton X-100 followed by PBS + 0.3% Triton X-100 and 2% normal goat serum for 2 hours with gentle rocking. Sections were then labeled with primary and secondary antibodies as described for cultured neurons.

Cultured neurons and brain sections were imaged on either of two microscopes: 1) Nikon Eclipse Ti equipped with a Yokogawa CSU-X1 spinning disk head, 20X 0.75 NA CFI Plan Apo, 40X 1.3 NA CFI S Fluor and 60x 1.4 NA Plan Apo objectives; 405 nm, 488 nm, 561 nm and 640 nm lasers; and a Hamamatsu Flash 4.0 scientific CMOS camera; or 2) EVOS FL imaging system (Invitrogen). Micrographs were captured using either Nikon Elements or EVOS software. CCR-positive cells were counted using the ImageJ cell counter plugin for brain sections (https://imagej.nih.gov/ij/plugins/cell-counter.html), and were counted manually using the EVOS microscope for primary neuron cultures. For cultured neurons (Figs. 1-4, S1 and S2), 4 independent, biological replicates were used per experiment, and for each independent replicate, >300 neurons were counted."

For brain sections (Fig. 5), 4 brains from each condition (WT with and without memantine, and Tg2576 with and without memantine) were used. To ensure thorough neuroanatomical coverage, 3 images for each of 3 cortical sections (lateral, medial, middle) were counted per brain to quantify cyclin D1-positive neurons. ~500 neurons per section were counted, yielding ~1,500 each of lateral, medial and middle cortical neurons, or a total of ~4,500 neurons per mouse. Because 4 brains per condition were counted in this manner, ~18,000 total neurons were counted for each condition. At a qualitative level, CCR neurons appeared to be evenly distributed throughout the cortical regions described here.

2.5. Western blotting

Samples were resolved on 12% SDS polyacrylamide gels, then transferred at 100 V for 1.75 hours onto 0.22 µm pore size nitrocellulose membranes using a Bio-Rad Mini-PROTEAN Tetra Electrophoresis Cell. Following transfer, blots were rinsed once in tris-buffered saline (TBS), blocked for 30-60 minutes in blocking buffer (LI-COR, 927-50000), sequentially incubated in primary antibodies overnight at 4° C in antibody buffer (1:1 mix of blocking buffer, and TBST: TBS +0.1% Tween-20), then LI-COR infrared-labeled secondary antibodies for 30 minutes. Blots were rinsed 3 times for 5 minutes each using TBST after the primary antibody or TBS after the secondary antibody. Finally, membranes were scanned using a LI-COR Odyssey imaging station.

2.6 Lentivirus production

HEK-293T cells were grown in culture to 90% confluency, then transfected with envelope, packaging, and shRNA plasmids using Lipofectamine 3000 (Invitrogen, L3000-015). Mission shRNAs for the knockdown vectors were obtained from Sigma-Aldrich (NR1 knockdown vector: TRCN0000233327). Media collected at 2-3 and 4-5 days post-transfection was pooled, and virus was pelleted in a Beckman Coulter Optima LE-80K ultracentrifuge for 2 hours at 23,000 rpm (95,389 x g_{max}) at 4° C in an SW28 rotor. The supernatant was then removed and the pellets containing the virus were resuspended in 300 µl Neurobasal medium. Viruses were then aliquoted and stored at -80° C until ready for use.

2.7. Memantine treatment

Tg2576 AD model mice that overexpress human β-amyloid precursor protein (APP) with the K670N/M67IL Swedish mutation [27] and WT mice of the same background (50% SLW, 50% C57/BL6) were treated with memantine at ~30 mg/kg/day based on a previously described protocol [28] from the time they were weaned (3 weeks) until 2 months of age. The memantine concentration in water, ~1 mM, chosen based on mouse weight and the assumption of mice consuming 0.15 ml of water per g body weight per day. Mice were given *ad libitum* access to food and water.

2.8. Statistics

All statistical analysis was performed with Prism 7 software. For BAPTA-AM, MK-801, memantine and knockdown experiments, one-way ANOVAs with Bonferroni post-hoc tests were used for analysis. For the phosphorylated calcium-calmodulin-dependent protein kinase II (pCaMKII) time course, a two-way ANOVA with Bonferroni post-hoc test was used to give p values for individual time points. For Tg2576 memantine experiments, one-way ANOVA with Bonferroni post-hoc test was used. At least 4,000 neurons were counted per mouse per condition.

2.9. Reagents used

Drugs: 2-Aminoethoxydiphenyl borate (2-ABP: Sigma; D9754-10G), BAPTA-AM (Thermo-Fisher; B1205), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX: Sigma; C239-100MG), dantrolene (Sigma; D9175-100MG), MK-801 (Sigma; M107-5MG), memantine (Sigma; M9292-100MG). Antibodies: chicken anti-MAP2 (Abcam; ab92434), mouse anti-NeuN (Millipore; MAB377), rabbit anti-CyclinD1 (Abcam; ab16663), rabbit anti-NR1 (CST; D65B7), rabbit anti-pCaMKII (CST; D21E4), mouse anti-total CaMKII (BD Labs; 611292), mouse anti-βIII-tubulin; (TuJ1;

courtesy of T. Spano and T. Frankfurter, University of Virginia), goat anti-mouse IgG Alexa Fluor 568 (LifeTechnologies; A11041), goat anti-mouse IgG Alexa Fluor 405 (Invitrogen; 35501BID), goat anti-rabbit IgG Alexa Fluor 488 (LifeTechnologies; A11034), goat anti-chicken IgG Alexa Fluor 647 (LifeTechnologies; A21235), goat anti-mouse IRDye 680LT (Licor; 925-68070), goat anti-rabbit IRDye 800 CW (Licor; 925-32211.

3. Results

3.1. Intracellular Calcium is Necessary for CCR

To test the hypothesis that neuronal CCR is calcium-dependent we first treated primary mouse cortical neurons with BAPTA-AM, a cell-permeant chelator of intracellular calcium, beginning 30 minutes prior to addition of AβOs to the cultures. After 16-18 hours of AβO exposure, neurons were fixed and stained by triple immunofluorescence microscopy with antibodies to the G1 marker, cyclin D1, the neuron-specific nuclear protein, NeuN, and the neuronal somatodendritic protein, MAP2. As shown in Fig. 1, AβO treatment increased the fraction of cyclin D1-positive neurons from ~8% to ~25%, but the rise in cyclin D1-positive neurons was prevented by pre-treatment with BAPTA-AM. These results demonstrate that calcium is necessary for neuronal CCR.

3.2. CCR is blocked by pharmacologically inhibiting NMDAR, but not AMPA receptor or ER calcium stores

We next investigated which specific calcium sources contribute to the initiation of CCR. Given the numerous deficits induced by AβOs at the synapse, and that AβOs induce calcium influx through NMDAR, we tested whether this AβO-mediated calcium influx is required for CCR. Accordingly, primary neuron cultures were treated with MK-801, an NMDAR inhibitor that blocks calcium entry through the channel pore, beginning 30 minutes before AβOs were added. After 16-18 hours of AβO treatment, neurons were fixed and stained for NeuN, MAP2 and cyclin D1 to enable quantitation of neuronal CCR. As shown in Fig. 2A/B, pre-treatment of neurons with MK-801 blocked the induction of CCR by AβOs. Otherwise identical experiments were performed using 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) to block plasma membrane AMPA receptors

(Fig. S1), 2-aminoethoxydiphenyl borate (2-ABP) to block ER-associated IP_3 receptors and TRP channels (Fig. S2), or dantrolene to inhibit ER-associated ryanodine receptors (Fig. S2). Because none of those inhibitors blocked CCR, we conclude that NMDAR is the major, and possibly exclusive source of the excess calcium that enters neurons in response to AβO exposure and initiates CCR.

Previous work from from our lab demonstrated that AβO-mediated CCR requires activation of at least four protein kinases: CaMKII, the src-family kinase, fyn, protein kinase A (PKA) and mTOR [19, 20]. CaMKII is of particular interest, as it can be activated via calcium influx through NMDAR. We thus tested the hypothesis that AβO-mediated calcium influx through NMDAR activates CaMKII as a mediator of CCR. Again, we treated primary neurons with AβOs with or without a 30 minute MK-801 pre-treatment, and collected the cells at various time points from 0-2 hours after AβO stimulation for western blotting with antibodies to phospho-activated and total CaMKII. As shown in Fig. 2C/D, we found that CaMKII was transiently activated 15 minutes after AβO addition, and that activation was prevented by MK-801. The AβO-mediated activation of CaMKII necessary for CCR [19] is therefore dependent upon calcium influx through NMDAR.

3.3. Knockdown of NR1 blocks CCR

To provide independent, non-pharmacological evidence for the role of NMDAR in AβO-stimulated neuronal CCR, we used antisense shRNA to knock down expression of the constitutive NMDAR subunit, NR1. 96 hours prior to AβO addition, primary neuron cultures were transduced with lentivirus containing an empty vector, or an shRNA vector to NR1. Following a 16-18 hour exposure to AβOs, the cells were processed for triple immunofluorescence with antibodies to

MAP2, NeuN and cyclin D1. As shown in Fig. 3, we found that reducing the neuronal level of NR1 to 30% of normal blocked the ability of AβOs to cause CCR. These results validate the pharmacological evidence based on the use of MK-801 (Fig. 2) that NMDAR is essential for enabling AβOs to drive normally post-mitotic neurons back into the cell cycle.

3.4. Memantine blocks CCR in cultured neurons and in vivo

Memantine is an FDA-approved drug for AD treatment, and works by preventing excess calcium influx through NMDAR while still allowing normal calcium-mediated synaptic transmission through the receptor. To gain further insight into the mechanism by which excess calcium induces CCR, we tested whether specifically blocking excess, but not normal calcium entry through NMDAR could block CCR. Similar to experiments with MK-801, we treated primary neurons with AβOs for 16-18 hours with or without a 30 minute pre-treatment with memantine, followed by triple immunofluorescence labeling with antibodies to NeuN, MAP2 and cyclin D1. As shown in Fig. 4, memantine, like MK-801 (Fig. 2), prevented AβOs from inducing neuronal CCR. Although memantine is typically used to treat late stage AD and is not considered to be a disease-modifying drug, these results with cultured neurons raised the possibility that memantine can interfere with CCR *in vivo* and might therefore be able to block neuron death in AD.

To test that possibility, wild type (WT) and Tg2576 AD model mice [27], which overexpress human APP with the Swedish mutation (K670M/N671L), were provided *ad libitum* access to memantine-containing water from the time they were weaned (3 weeks) until 2 months of age, when abundant neuronal CCR is normally evident in the Tg2576 strain [20]. Following the 5 weeks of memantine treatment, the animals were euthanized and brain sections were stained with

antibodies to NeuN, cyclin D1 and the neuron-specific protein, βIII-tubulin. As shown in Fig. 5, WT mice with or without memantine treatment had a basal level of 1.6% neuronal CCR along cortical regions. as determined by cyclin D1 immunoreactivity. In contrast, the basal level of CCR in similar cortical regions of Tg2576 mice was 8.8%, which was reduced to WT levels by memantine. These results show that treating Tg2576 mice with memantine before symptom onset acts prophylactically to prevent CCR.

4. Discussion

The behavioral symptoms of AD are directly caused by the impaired function and loss of synapses by neurons that control memory and cognition, and by the death of those neurons. Using CCR as a surrogate for eventual neuronal death in primary neuron cultures and *in vivo*, we demonstrate here that AβO-stimulated entry of excess calcium via NMDAR, which has been shown by others to trigger synaptotoxicity *in vivo* [3,29,30], also initiates neuronal CCR. We found that AβO-induced CCR could be prevented in cultured neurons by chelating total intracellular calcium with BAPTA-AM (Fig. 1), blocking calcium entry via NMDAR with MK-801 (Fig. 2), shRNA knockdown of the constitutive NMDAR subunit NR1 (Fig. 3), or using memantine for specifically blocking the entry of excess calcium into neurons via NMDAR (Fig. 4). In contrast, inhibitors of cytoplasmic calcium elevation by AMPA receptor (Fig. S1) or ER calcium stores (Fig. S2) did not block AβO-induced CCR. Most importantly in terms of clinical relevance, we also show that memantine treatment of Tg25756 AD model mice blocks neuronal CCR *in vivo* (Fig. 5). Together, these results indicate that AβO-stimulated excitotoxic calcium influx through NMDAR and CCR/cell death share a common mechanistic origin. Furthermore, they suggest that memantine, which is widely considered to relieve symptoms without attacking processes that lead to neuronal decline, may indeed slow or prevent AD progression if administered at early, pre-symptomatic disease stages.

While the pathways for both synaptotoxicity [11, 32] and CCR/cell death proceed following AβO-stimulated entry of excess calcium via NMDAR, it is not yet clear if these dual causes of AD symptoms are connected serially or represent bifurcating processes with a common origin. As illustrated in Fig. 6, AβO-mediated synaptic deficits and CCR stemming from aberrant NMDAR calcium influx could be connected by a linear pathway in either direction, or

alternatively, AβO-mediated calcium influx could contribute to synaptic deficits and CCR independently, eventually causing neuron death. These two schemes are not necessarily mutually exclusive, and further work will be needed to resolve this issue. Although CCR neurons are abundant in Tg2576 mice by 2 months of age [20] and the earliest reported synaptic problem in this strain is dendritic spine loss by 4.5 months of age [33], it is possible that synaptic deficits that are difficult to detect in Tg2576 mice occur before CCR or contribute to CCR at later time points.

It has been known since the 1990s that vulnerable neurons in AD frequently enter into a paradoxical pathway before dying: ectopic CCR. Differentiated neurons are permanently post-mitotic, but in AD and several other neurodegenerative disorders, neuron death often appears to follow expression of various cell markers, such as cyclin D1 and duplication of large portions of the genome [18,31]. Furthermore, studies of human brain have shown a peak in polyploidy between preclinical AD and mild AD, followed by a decline in polyploidy and neuronal numbers in severe AD, presumably because CCR neurons preferentially die [17].

AβOs are the dominant species causing the excess calcium influx that contributes to excitotoxicity in AD, particularly through NMDARs [33,34]. The data shown here imply that excitotoxicity and CCR are mechanistically connected to NMDAR activity by tau. For instance, tau plays an essential role in effects downstream of excitotoxic calcium influx, as knocking out tau in an AD model mouse model protects against learning and memory deficits, seizures and premature death [35]. Additionally, it has been shown that NMDAR can be influenced by tau upstream of calcium influx, by recruiting the non-receptor tyrosine kinase, fyn, which phosphorylates NMDAR and thereby facilitates excess calcium influx provoked by AβOs [3,29,36]. We have also shown tau to be necessary for CCR to occur by a mechanism that requires

fyn-dependent tau phosphorylation [19]. Although our results imply that excess calcium entry into neurons via NMDAR is sufficient to drive CCR, they do not exclude possible contributions to cytoplasmic calcium level increases from at least one other source, mitochondria [37].

There are additional ways that calcium is likely to be connected to CCR. Calcium is one of the most versatile second messengers [38], not only for controlling functions of post-mitotic neurons, but also in regulating division of proliferative cells. In fact, calcium is involved at multiple stages of the cell cycle, including conversion from the quiescent state of G0 to G1, and from G1 to S phase [39,40]. These transitions are germane to neuronal CCR, because neurons undergoing CCR apparently proceed into S phase before their eventual death. In other excitotoxic paradigms, such as stroke and ischemia, NMDAR overexcitation by kainic acid treatment is sufficient to induce CCR in neurons before their eventual death by excitotoxic shock [41-43]. It is possible that this already established pathway has been repurposed in the neuron as a response to stress or toxicity.

Memantine alleviates many AD symptoms, including loss of LTP/LTD, learning and memory deficits [28,44], synapse loss, and abnormal tau phosphorylation [45]. Memantine is additionally protective against more canonic excitotoxic insults beyond AD, including preventing neuron damage after ischemic shock in mice and rats [46,47]. Our results show that memantine also works prophylactically to prevent CCR when used before disease signs are evident. This raises the possibility that memantine has disease-modifying properties that have not been realized until now, and that it might slow or halt disease progression if administered at a sufficiently early pre-symptomatic disease stage.

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Figure legends

Fig. 1. Intracellular calcium chelation by BAPTA-AM prevents AβO-induced neuronal CCR. (A) Primary cortical neurons were treated for 18 hours with AβOs with or without 10 μM BAPTA-AM to chelate intracellular calcium. CCR neurons were identified by their immunoreactivity with antibodies to both cyclin D1, which marks nuclei during G1 of the cell cycle, and NeuN, which marks nuclei in all neurons. Neuronal somatodendritic compartments were labeled with an antibody to MAP2. (B) Quantification of the immunofluorescence results and statistical analysis by one-way ANOVA. Error bars indicate s.e.m.

Fig. 2. The NMDAR inhibitor, MK-801, blocks AβO-induced neuronal CCR and early activation of CaMKII. (A) Primary cortical neurons were treated overnight with AβOs with or without 10 μM MK-801. After 18 hours of exposure to AβOs, neurons were stained for MAP2, NeuN, and Cyclin D1 to mark neurons that re-enter the cell cycle. (B) Quantification of the immunofluorescence results and statistical analysis by one-way ANOVA. Error bars indicate s.e.m. (C) Primary cortical neurons were treated for the indicated times with AβOs, with or without 10 μM MK-801, after which phospho-activated and total CaMKII levels were monitored by western blotting. (D) Quantification of phosphorylated CaMKII relative to total CaMKII at each time point. Note that MK-801 prevented the transient rise in phospho-activation of CaMKII. Results are significant by two-way ANOVA when comparing the plus versus minus MK-801 data sets ($p < 0.005$), and by Bonferroni post hoc analysis when comparing the 0 and 15-minute time points without MK-801 ($p < 0.01$), and when comparing plus and minus MK-801 at the 15 minute time point ($p < 0.0001$). Error bars indicate s.e.m.

Fig. 3. Knockdown of the NR1 subunit of NMDAR prevents AβO-induced neuronal CCR. (A) Primary cortical neurons were transduced for 96 hours prior to AβO addition with lentivirus expressing shRNA to NR1, or as a control, with lentivirus comprising an empty expression vector. After 16-18 hours of AβO exposure, the cells were stained by triple immunofluorescence for NeuN and MAP2 to mark neurons, and for cyclin D1 to assess CCR. (B) Quantification of the immunofluorescence results. Quantification of the immunofluorescence results and statistical analysis by one-way ANOVA. Error bars indicate s.e.m. C) Quantitative western blot showing a 30% knockdown of NR1.

Fig. 4. Memantine blocks AβO-induced neuronal CCR. (A) Primary cortical neurons were treated with AβOs with or without pre-treatment with 10 μM memantine. Cells were then stained with antibodies to MAP2, NeuN, and cyclin D1 to identify CCR neurons. (B) Quantification of the immunofluorescence results and statistical analysis by one-way ANOVA. Error bars indicate s.e.m.

Fig. 5. Treatment of Tg2576 AD model mice with memantine prevents CCR *in vivo*. (A) Tg2576 and wild type mice were provided *ad libitum* access to drinking water with or without memantine from the time of weaning at 3 weeks until 2 months of age. Brain sections from each condition were stained by triple immunofluorescence for the neuron-specific protein, βIII-tubulin, cyclin D1, and NeuN to identify CCR positive neurons. (B) Quantification of the immunofluorescence results and statistical analysis by one-way ANOVA. Error bars indicate s.e.m.

Fig. 6. AβO-induced calcium influx via NMDAR is necessary for neuronal CCR. AβO-induced excitotoxicity contributes to many neuronal dysfunctions in AD, including synaptic deficits. Here, we show that AβO-mediated calcium influx via NMDAR is also necessary for initiating CCR. AβO-mediated synaptic dysfunction involving NMDAR could be initiating neuronal CCR directly, or alternatively, these pathways could diverge after AβO-mediated calcium influx, each contributing to neuron death independently. These two schemes are not necessarily mutually exclusive.

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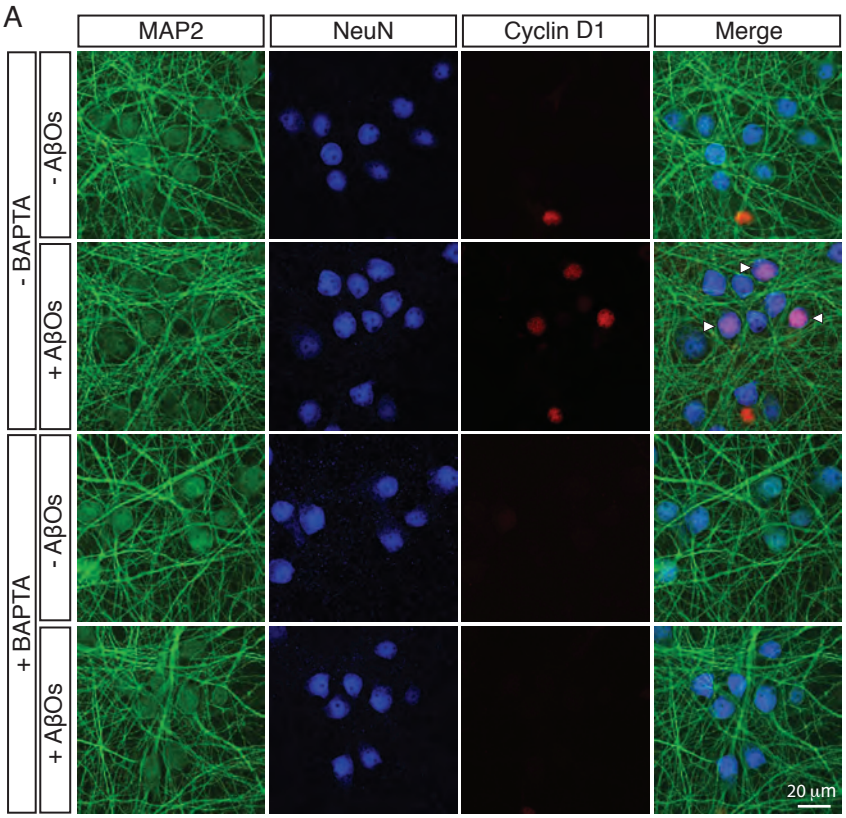

A
MAP2
NeuN
Cyclin D1
Merge
- BAPTA
+ BAPTA
- AβOs
+ AβOs
- AβOs
+ AβOs
20 μm

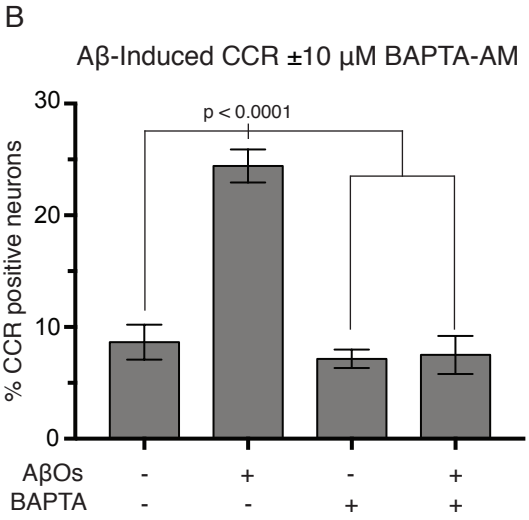
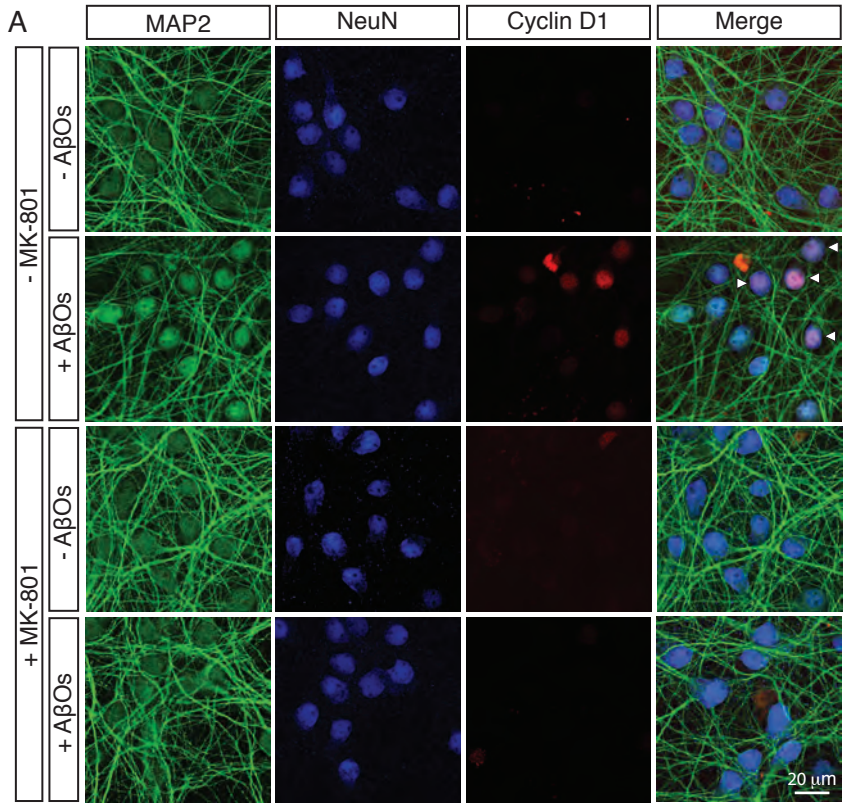
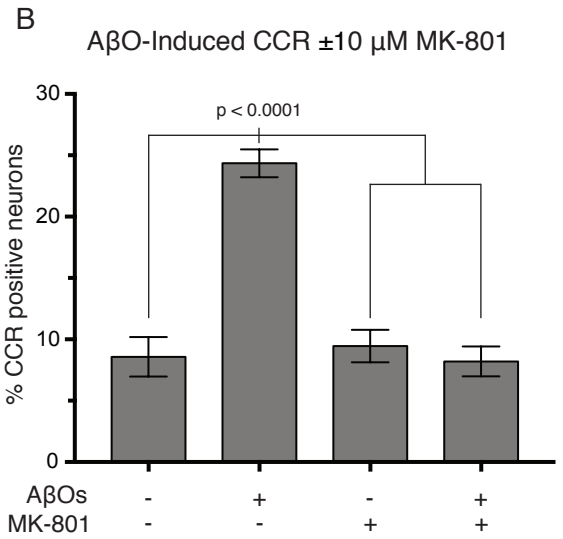
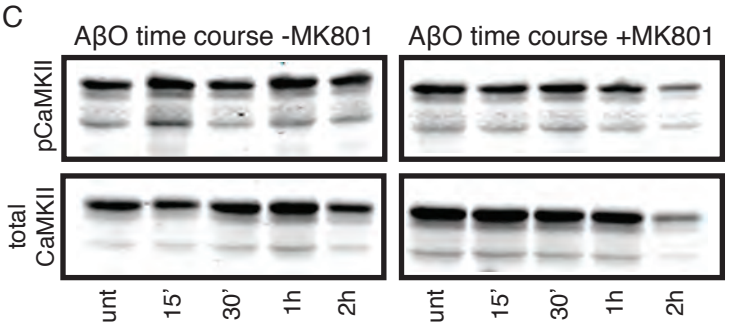

B
Aβ-Induced CCR ±10 μM BAPTA-AM
p < 0.0001
% CCR positive neurons
30
20
10
0
AβOs - + - +
BAPTA - - + +

Figure 1


A
MAP2
NeuN
Cyclin D1
Merge
- MK-801
+ MK-801
- AβOs
+ AβOs
20 μm


B
AβO-Induced CCR ±10 μM MK-801
% CCR positive neurons
p < 0.0001
AβOs
MK-801


C
AβO time course -MK801
AβO time course +MK801
pCaMKII
total CaMKII
unt
15'
30'
1h
2h

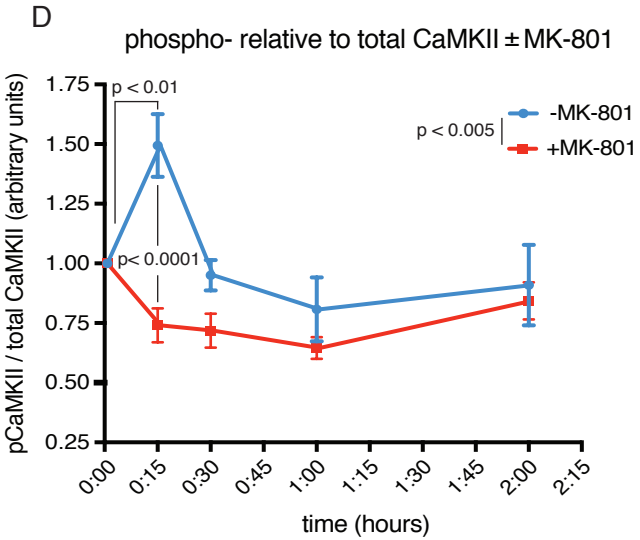
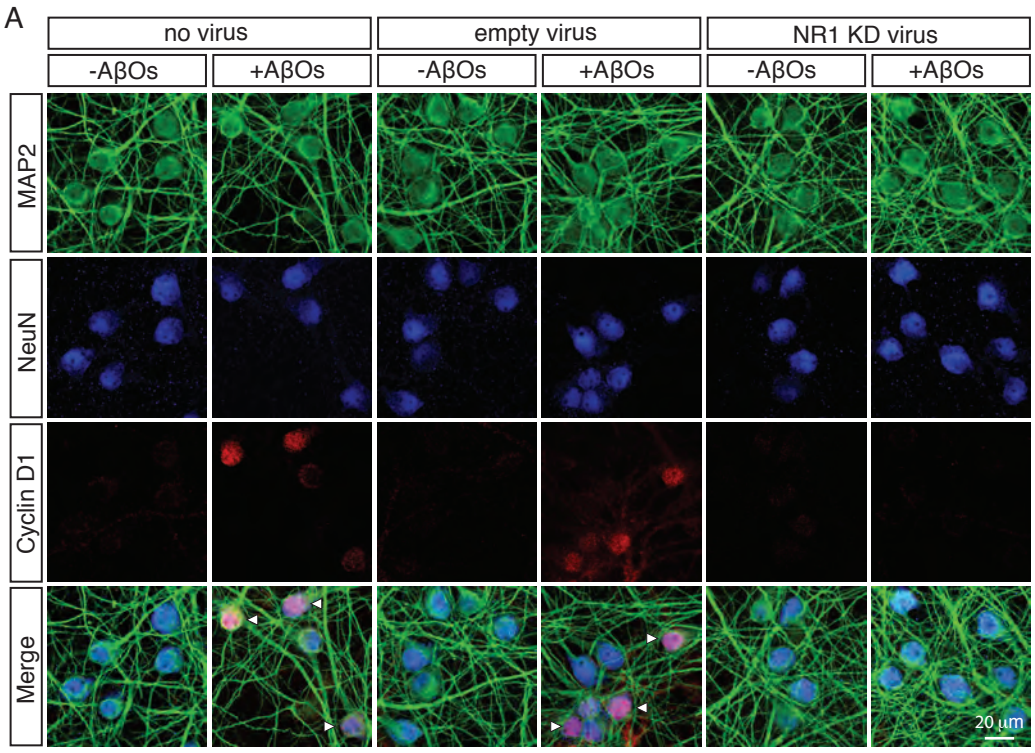
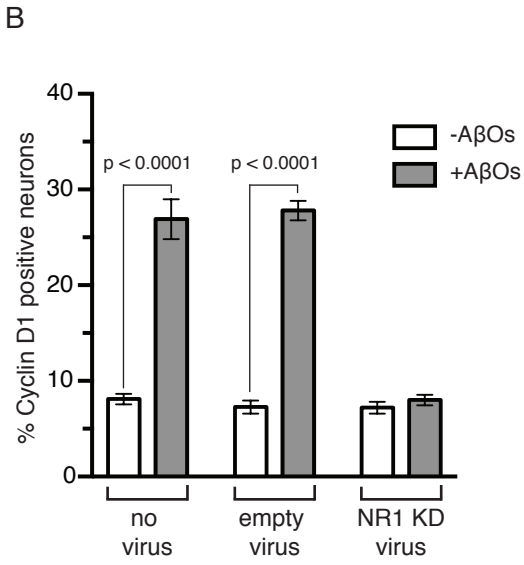

D
phospho- relative to total CaMKII ± MK-801
pCaMKII / total CaMKII (arbitrary units)
p < 0.01
p < 0.005
p< 0.0001
-MK-801
+MK-801
time (hours)

Figure 2


A
no virus
empty virus
NR1 KD virus
-AβOs
+AβOs
-AβOs
+AβOs
-AβOs
+AβOs
MAP2
NeuN
Cyclin D1
Merge
20 μm


B
% Cyclin D1 positive neurons
40
30
20
10
0
p < 0.0001
p < 0.0001
-AβOs
+AβOs
no virus
empty virus
NR1 KD virus

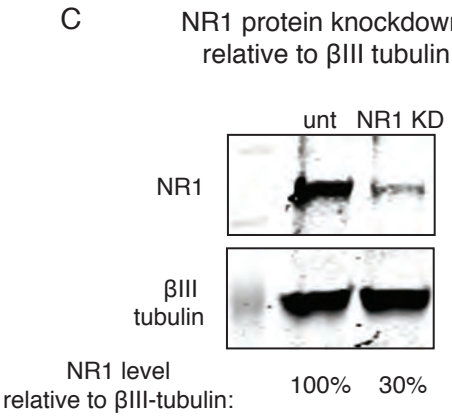
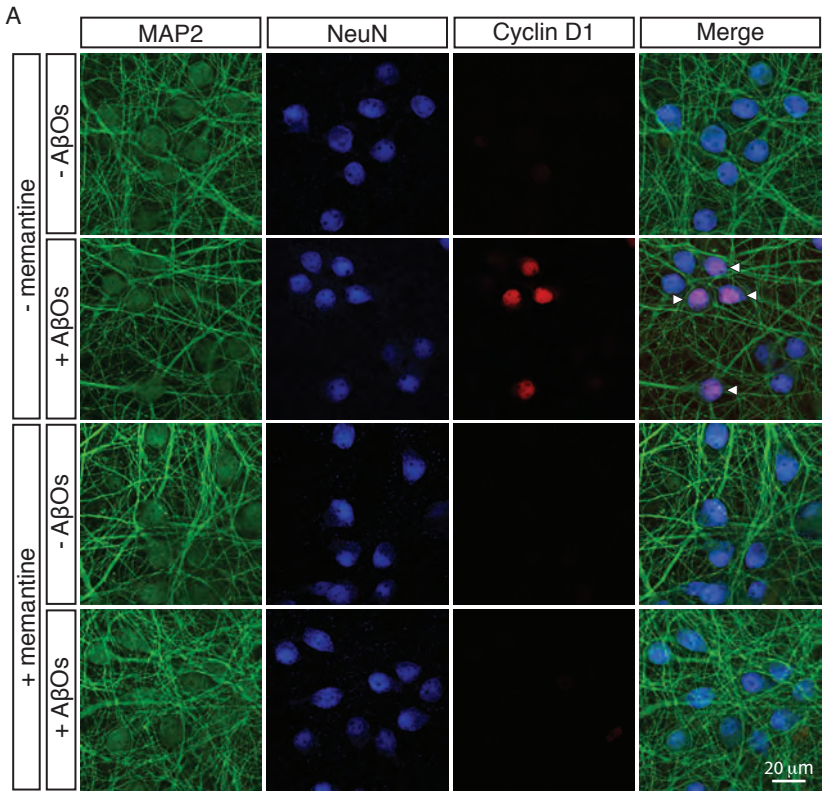

C
NR1 protein knockdown relative to βIII tubulin
unt
NR1 KD
NR1
βIII tubulin
NR1 level relative to βIII-tubulin:
100%
30%

Figure 3


A
MAP2
NeuN
Cyclin D1
Merge
- memantine
+ memantine
- AβOs
+ AβOs
- AβOs
+ AβOs
20 µm

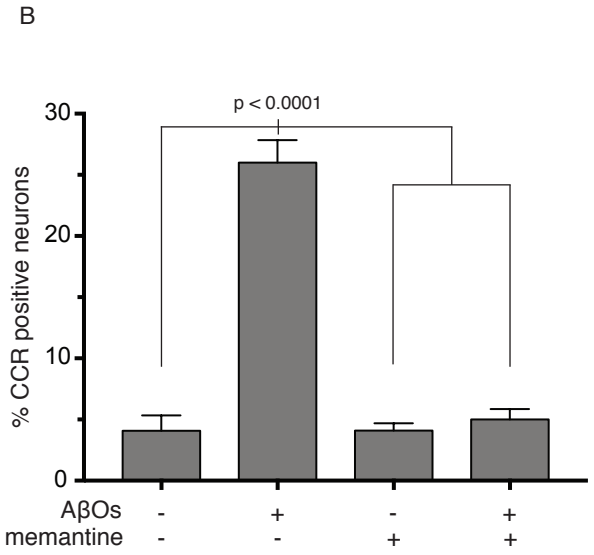
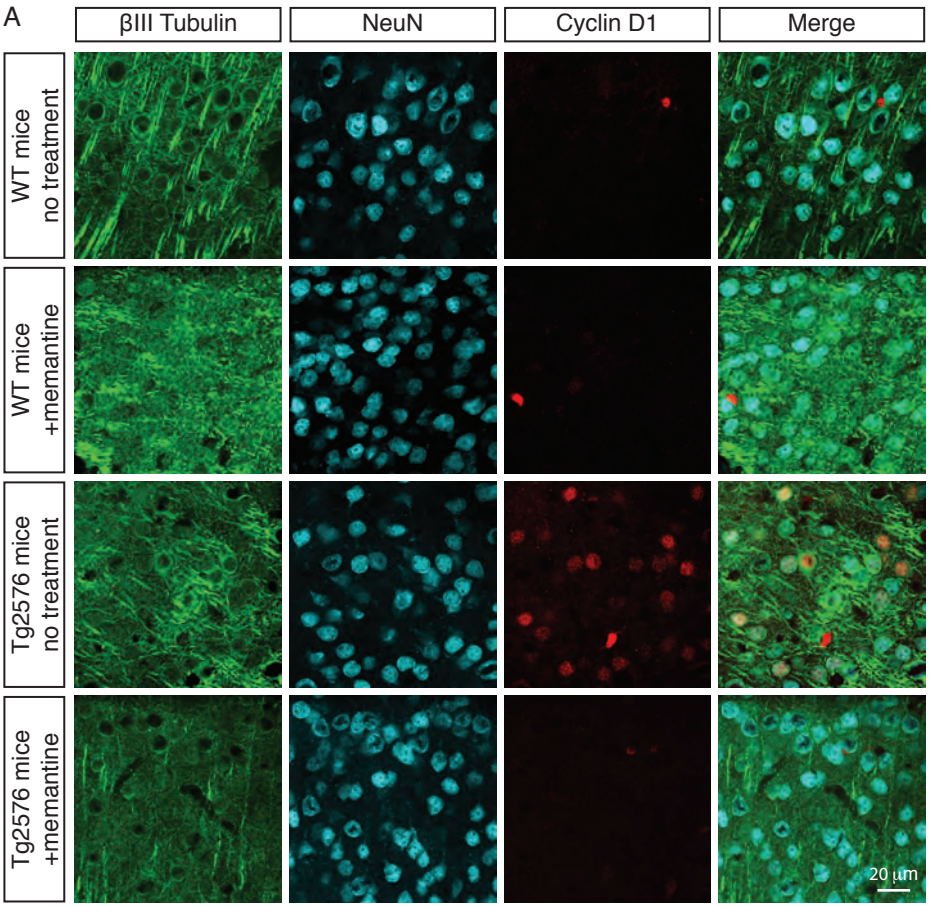

B
p < 0.0001
% CCR positive neurons
30
20
10
0
AβOs - + - +
memantine - - + +

Figure 4


A
βIII Tubulin
NeuN
Cyclin D1
Merge
WT mice no treatment
WT mice +memantine
Tg2576 mice no treatment
Tg2576 mice +memantine
20 µm

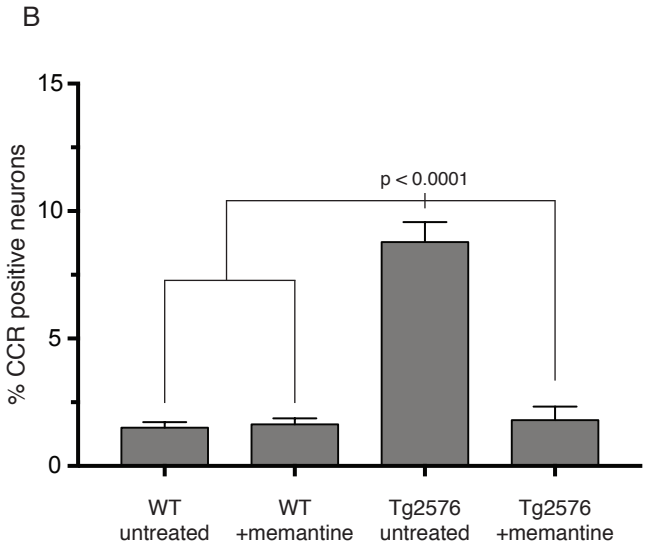

B
% CCR positive neurons
15
10
5
0
p < 0.0001
WT untreated
WT +memantine
Tg2576 untreated
Tg2576 +memantine

Figure 5

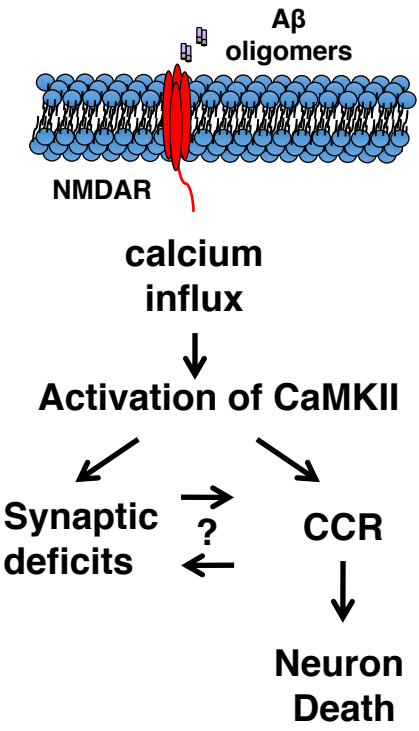

Aβ
oligomers
NMDAR
calcium
influx
Activation of CaMKII
Synaptic
deficits
?
CCR
Neuron
Death

Figure 6

Supplemental Material:

Fig. S1:

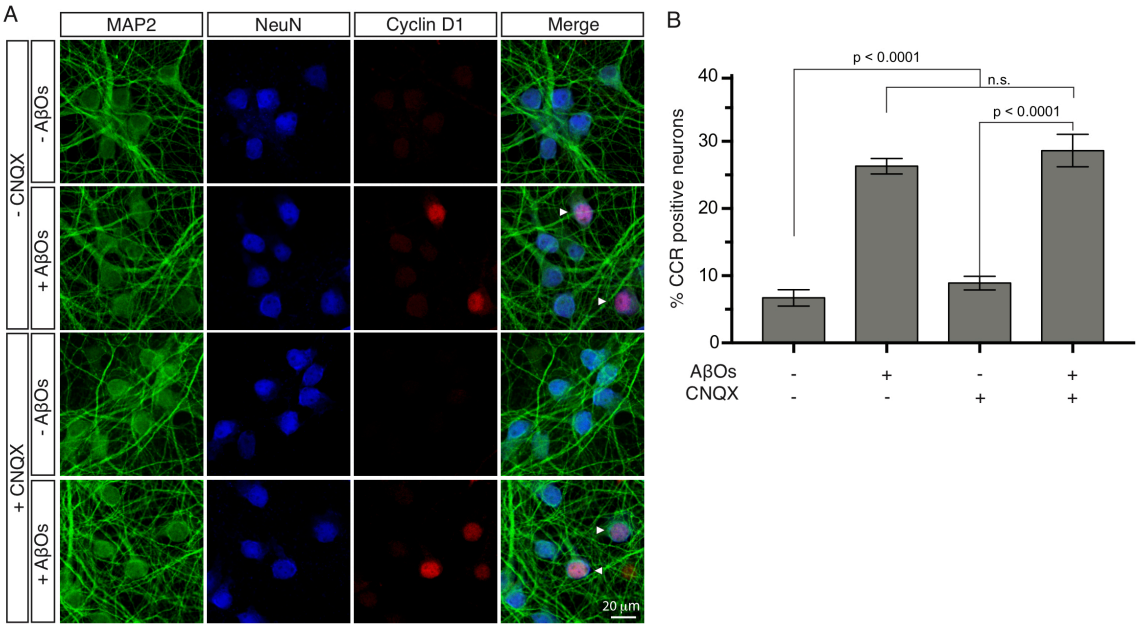


Fig. S1: The selective α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) inhibitor, CNQX, does not inhibit CCR. (A) Primary cortical neurons were treated overnight with AβOs with or without 10 μM CNQX. After 18 hours of exposure to AβOs, neurons were stained for MAP2, NeuN, and Cyclin D1 to mark neurons that had re-entered the cell cycle. (B) Quantification of the immunofluorescence results and statistical analysis by one-way ANOVA demonstrates that AMPAR inhibiton by CNQX does not block AβO-induced CCR. AMPAR therefore does not appear to modulate the AβO-induced calcium entry that drives CCR. Error bars indicate s.e.m.

Supplemental Fig. 2:

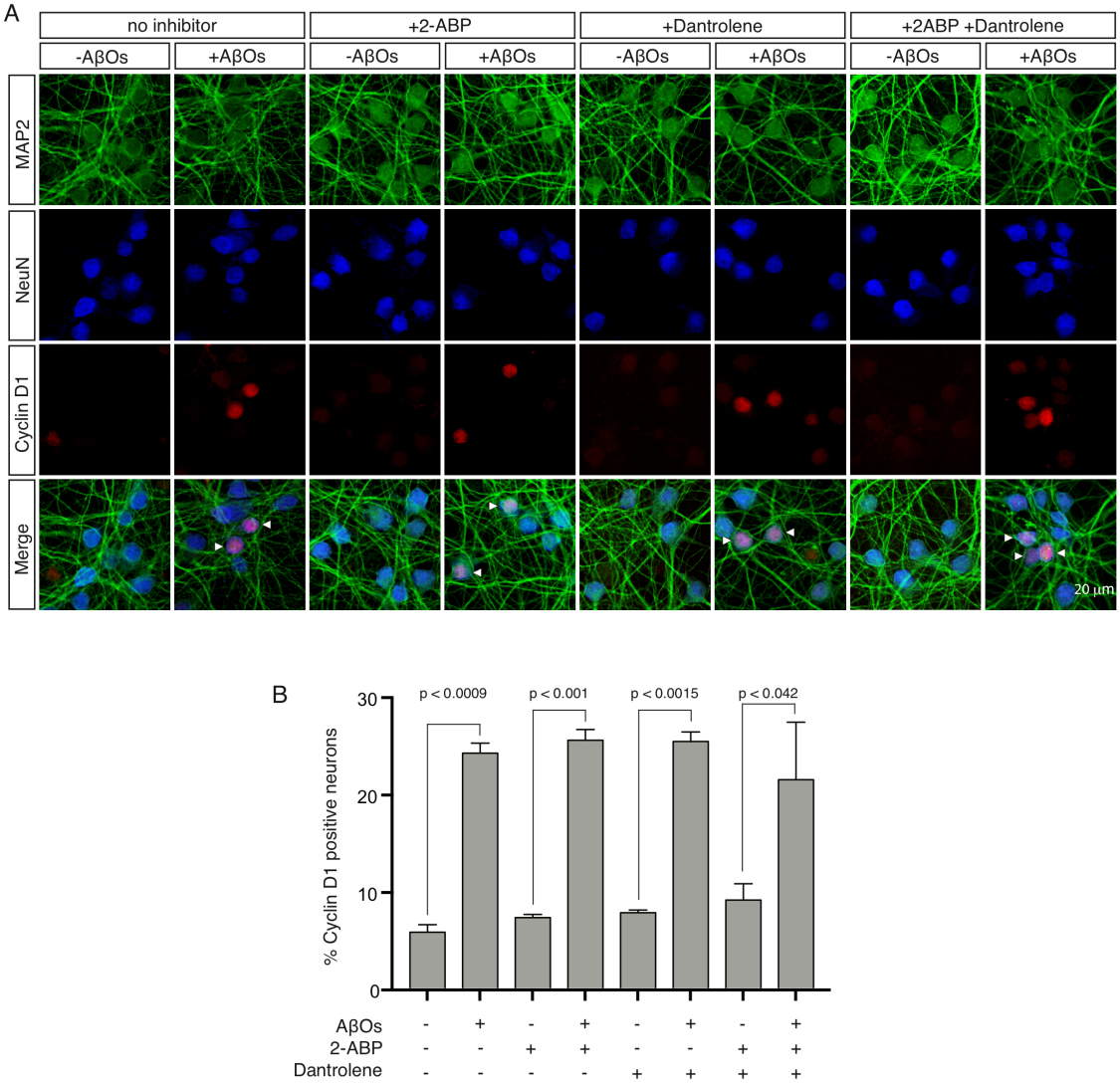


Fig. S2: Inhibitors of endoplasmic reticulum (ER) IP_3 and ryanodine receptors do not block activation of CCR. (A) Primary cortical neurons were treated overnight with AβOs with or without 50 µM 2-aminoethoxydiphenyl borate (2-APB) to block IP_3 receptors, 20 µM dantrolene to block ryanodine receptors, or both inhibitors. After 18 hours of exposure to AβOs, neurons were stained for MAP2, NeuN, and Cyclin D1 to mark neurons that had re-entered the cell cycle. (B) Quantification of the immunofluorescence results. Neither drug, alone or in combination, blocked AβO-induced CCR. Calcium release by the ER therefore does not contribute significantly to the AβO-induced calcium release that drives CCR. Indicated p values were calculated by one-way ANOVA using the Bonferroni post-hoc test. Error bars indicate s.e.m.