

Calcium flux-independent NMDA receptor activity is required for A β oligomer-induced synaptic loss

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Synaptic loss is one of the major features of Alzheimer's disease (AD) and correlates with the degree of dementia. *N*-methyl-D-aspartate receptors (NMDARs) have been shown to mediate downstream effects of the β -amyloid peptide (A β) in AD models. NMDARs can trigger intracellular cascades via Ca²⁺ entry, however, also Ca²⁺-independent (metabotropic) functions of NMDARs have been described. We aimed to determine whether ionotropic or metabotropic NMDAR signaling is required for the induction of synaptic loss by A β . We show that endogenous A β as well as exogenously added synthetic A β oligomers induced dendritic spine loss and reductions in pre- and postsynaptic protein levels in hippocampal slice cultures. Synaptic alterations were mitigated by blocking glutamate binding to NMDARs using NMDAR antagonist APV, but not by preventing ion flux with Ca²⁺ chelator BAPTA or open-channel blockers MK-801 or memantine. A β increased the activity of p38 MAPK, a kinase involved in long-term depression and inhibition of p38 MAPK abolished the loss of dendritic spines. A β -induced increase of p38 MAPK activity was prevented by APV but not by BAPTA, MK-801 or memantine treatment highlighting the role of glutamate binding to NMDARs but not Ca²⁺ flux for synaptic degeneration by A β . We further show that treatment with the G protein inhibitor pertussis toxin (PTX) did not prevent dendritic spine loss in the presence of A β oligomers. Our data suggest that A β induces the activation of p38 MAPK and subsequent synaptic loss through Ca²⁺ flux- and G protein-independent mechanisms.

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Alzheimer's disease (AD) is clinically characterized by cognitive impairments caused by massive neuronal degeneration and synaptic loss. The reduction in synapse numbers is the best neuropathological correlate to the degree of dementia in AD.¹ Besides synaptic alterations, the levels of soluble oligomeric forms of β -amyloid peptide (A β) but not plaques correlates best with memory loss in AD.² Accumulating evidence indicates that transgenically produced A β or the treatment with A β oligomers decrease dendritic spine density,^{3–6} impair long-term potentiation (LTP),⁷ facilitate long-term depression (LTD)⁸ and induce aberrant spine morphology.^{5,9}

Although the signaling cascades coupling A β with synaptic degeneration are incompletely understood, experimental evidence suggests an essential role for *N*-methyl-D-aspartate receptors (NMDARs). Oligomeric A β can bind to dendritic spines and treatment with NMDAR antibodies abolishes A β binding.¹⁰ Pharmacological inhibition of NMDAR activity also mitigates the pathological effect of A β on synapses.^{4–6,11} NMDARs are ionotropic receptors permeable for cations and controlled by a voltage-dependent Mg²⁺ block that is removed after membrane depolarization by α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA receptors). Upon glutamate binding to the NR2 subunit of NMDARs, cations including Ca²⁺ enter the cell. It has been thought for a long time that the levels of Ca²⁺ influx through NMDARs determine the induction of either LTP (high Ca²⁺ influx or LTD (mild Ca²⁺

influx).¹² Nevertheless, a recent study showed that Ca²⁺ flux is not essential for the induction of NMDAR-LTD, whereas glutamate binding to the receptor is required.¹³ NMDAR signaling independently of ion flux has already been proposed to regulate NMDAR phosphorylation and endocytosis.^{14,15} Further, the subunit switch between NR2B and NR2A NMDARs is driven by glutamate in the absence of NMDAR currents.¹⁶ However, the role of ion flux for synaptic loss in AD still remains to be elucidated.

We show that the A β -induced pre- and postsynaptic loss is mediated by glutamate binding to NMDARs, independent of ion influx.

Results

To determine the mechanisms of synaptic loss by A β , we cultured hippocampal slices from arcA β -transgenic mice and infected them with neurotropic Sindbis virus expressing EGFP to visualize single neurons. Neurons in transgenic slices showed reduced dendritic spine densities. Treatment with the NMDAR antagonist D-APV, which blocks the glutamate-binding sites, completely abolished spine loss (Figures 1a and b). This is in agreement with previous findings that the glutamate-binding site antagonist CPP rescued spine loss in APP-transgenic cultures.⁵ As NMDAR signaling has been reported to be mediated through Ca²⁺ influx, we sought to

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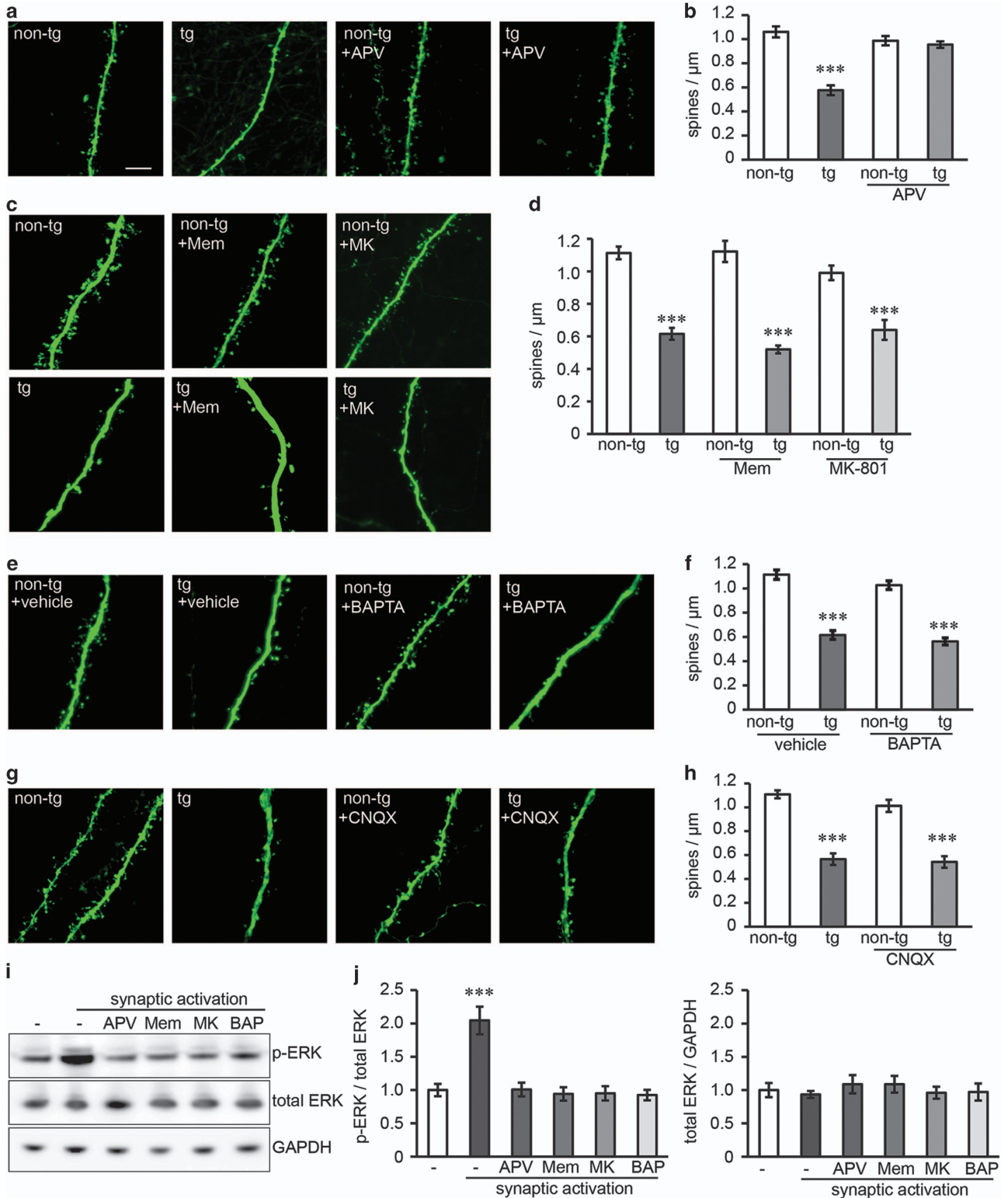
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Abbreviations: AD, Alzheimer's disease; A β , β -amyloid peptide; NMDAR, N-methyl-D-aspartate receptor; LTD, long-term-depression; PTX, Pertussis toxin; p-ERK, phospho-ERK

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determine the role of Ca²⁺ influx for A β -induced loss of dendritic spines. To this end, slices were treated with NMDAR open-channel blockers memantine or MK-801 (Figure 1c) or with Ca²⁺ chelator 1,2-bis(2-aminophenoxy)ethane-*N,N,N,N'*-

tetraacetic acid (BAPTA; Figure 1e). Neither memantine nor MK-801 nor BAPTA treatment restored spine density in transgenic cultures (Figures 1d and f). Ca²⁺ flux through NMDARs requires the removal of the Mg²⁺ block within the



receptor pore, which is achieved upon membrane depolarization by AMPARs. To analyze the involvement of AMPARs, we treated cultures with AMPAR antagonist CNQX, but did not observe any effect on spine numbers in transgenic cultures (Figures 1g and h). Concentrations of inhibitors were chosen according to previous reports demonstrating highest degree of specificity and/or therapeutic relevance (APV and MK-801;¹⁷ memantine^{18,19}).

To ensure that memantine, MK-801 and BAPTA preparations at the used concentrations are functional and can block NMDARs and the entry of Ca²⁺, despite having no protective effect, we performed synaptic activation experiments in the presence of these compounds. Synaptic activation induced phosphorylation of extracellular signal regulated kinases (phospho-ERK, p-ERK), which is in agreement with previous studies^{6,20} (Figure 1i). The presence of APV, memantine, MK-801 or BAPTA fully prevented ERK activation, confirming the functionality of these compounds (Figure 1j). Of note, memantine blocked synaptic activation although it has been described to preferentially block extrasynaptic over synaptic activity at the used concentration.²¹

As shown previously, spine loss in arcA β -transgenic cultures can be prevented in the presence of anti-A β antibodies,⁶ confirming that A β but not APP or any other cleavage product is responsible for the observed effects on spines. Hence, our data indicate that glutamate binding to NMDARs rather than Ca²⁺ flux mediates A β -induced dendritic spine loss.

To confirm the morphological spine data, we determined whether A β also affects the levels of pre- and postsynaptic proteins and analyzed PSD-95 (postsynaptic) and synaptophysin (presynaptic) levels in lysates of non-transgenic and arcA β -transgenic cultures (Figure 2a). Compared with controls, the levels of both proteins were strongly reduced in transgenic cultures. Treatment with APV but not with memantine or MK-801 rescued the reduction of protein levels (Figure 2b). Likewise, treatment with BAPTA did not affect loss of synaptic proteins in transgenic cultures (Figures 2c and d).

Synaptic activity has been shown to increase the production of A β and, inversely, preventing synaptic activity can reduce A β production.²² To exclude that the protective effect of APV is simply based on A β reduction, we quantitatively measured the levels of A β 40 in the supernatant of arcA β -transgenic cultures treated with the respective NMDAR antagonists (Figure 2e) under identical conditions as in the previous experiments. NMDAR antagonists did not significantly alter A β levels in the medium of transgenic cultures.

This indicates that, in addition to dendritic spine loss, reductions in pre- and postsynaptic protein levels are caused by NMDAR functions, independent of Ca²⁺ flux.

A recent study showed that activation of p38 MAPK is essential for Ca²⁺-independent metabotropic function of NMDARs.¹³ We analyzed whether p38 MAPK is also involved in the A β effects on synapses and examined the activity of p38 MAPK in lysates from non-transgenic and arcA β -transgenic cultures (Figure 3a). Increased levels of phosphorylated (active) p38 MAPK (p-p38) were observed in transgenic cultures. After treatment with APV, the levels of p-p38 were reduced to control levels, whereas memantine or MK-801 treatment had no effect (Figure 3b). To ascertain that active p38 MAPK mediates synaptic deficits caused by A β , we treated cultures with the p38 MAPK inhibitor SB239063. Treatment abolished spine loss in arcA β -transgenic cultures (Figures 3c and d). To examine whether a general increase in synaptic activity in transgenic cultures contributes to increased p-p38 MAPK levels, we treated non-transgenic and arcA β -transgenic cultures with bicuculline and 4-aminopyridine (Figures 3e and f). Synaptic activation increased levels of p-ERK. No difference in p-ERK levels were observed between transgenic and non-transgenic cultures, indicating that arcA β -transgenic slices display no general increase in synaptic activity. Further, synaptic activation does not affect the activity of p38 MAPK.

This data suggests that A β induces the activity of p38 MAPK, which mediates the loss of dendritic spines. This effect does not depend on Ca²⁺ influx or general synaptic activation.

Oligomeric A β is considered to be one of the main toxic A β species in the AD brain. So far, we used cultures from arcA β -transgenic mice to determine the effects of A β on synapses in the presence of other APP processing products (Figures 1–3). Although arcA β mice show early formation oligomeric A β *in vivo*,²³ the role of oligomeric A β for our findings requires further investigations. To conclusively validate the role of A β oligomers, we treated non-transgenic cultures with defined preparation of A β 42 oligomers at sublethal concentrations (Figure 4). Oligomer preparations contained mostly mono-, tri- and tetramers as determined by silver stained SDS gel and western blot (Figure 4k), which is in agreement with previous studies.^{24,25} Scrambled A β , subjected to the same oligomerization protocol as A β 42, did not aggregate, as expected. Treatment with A β oligomers but not scrambled A β reduced dendritic spine density to a similar extent as observed in transgenic cultures (compare Figure 4 and Figure 1). Confirming the transgenic data, only APV treatment (Figures 4a and b) but not memantine (Figures 4c and d), MK-801 (Figures 4e and f) or BAPTA (Figures 4g and h) prevented

Figure 1 Blocking glutamate binding to NMDARs but not Ca²⁺ influx prevents dendritic spine loss in arcA β -transgenic slice cultures. (a) Confocal images of dendrites from CA1 neurons in the stratum radiatum of non-transgenic and arcA β -transgenic hippocampal slice cultures treated with NMDAR antagonist APV (100 μ M). Scale bar: 5 μ m. (b) APV treatment reverses the dendritic spine loss in arcA β -transgenic cultures. $n = 10$ –13. (c) Confocal images of non-transgenic and arcA β -transgenic cultures treated with NMDAR open-channel blocker memantine (1 μ M) or MK-801 (30 μ M). (d) Neither memantine nor MK-801 treatment reverses spine loss. $n = 11$ –13. (e) Confocal images of cultures treated with Ca²⁺ chelator BAPTA (2 mM) or vehicle (BAPTA solvent NaHCO₃). (f) BAPTA treatment does not affect spine loss in transgenic cultures. (g) Confocal images of cultures treated with AMPAR antagonist CNQX (10 μ M). (h) CNQX treatment does not affect spine loss in transgenic cultures. $n = 11$ –15. (i) Western blot of lysate from non-transgenic cultures after synaptic activation—in the presence of the reagents used above—showing phosphorylated and total ERK levels. (j) APV (100 μ M), memantine (1 μ M), MK-801 (30 μ M) and BAPTA (2 mM) pre-treatment prevent ERK phosphorylation after synaptic activation. All values are shown as mean \pm S.E.M.; *** $P < 0.001$; two-tailed unpaired Student's *t*-test; significances show difference to the respective non-transgenic control (b–h) or to non-activated cultures (j). non-tg, non-transgenic; tg, arcA β transgenic; Mem, memantine; BAP, BAPTA; MK, MK-801; p-ERK, phospho-ERK

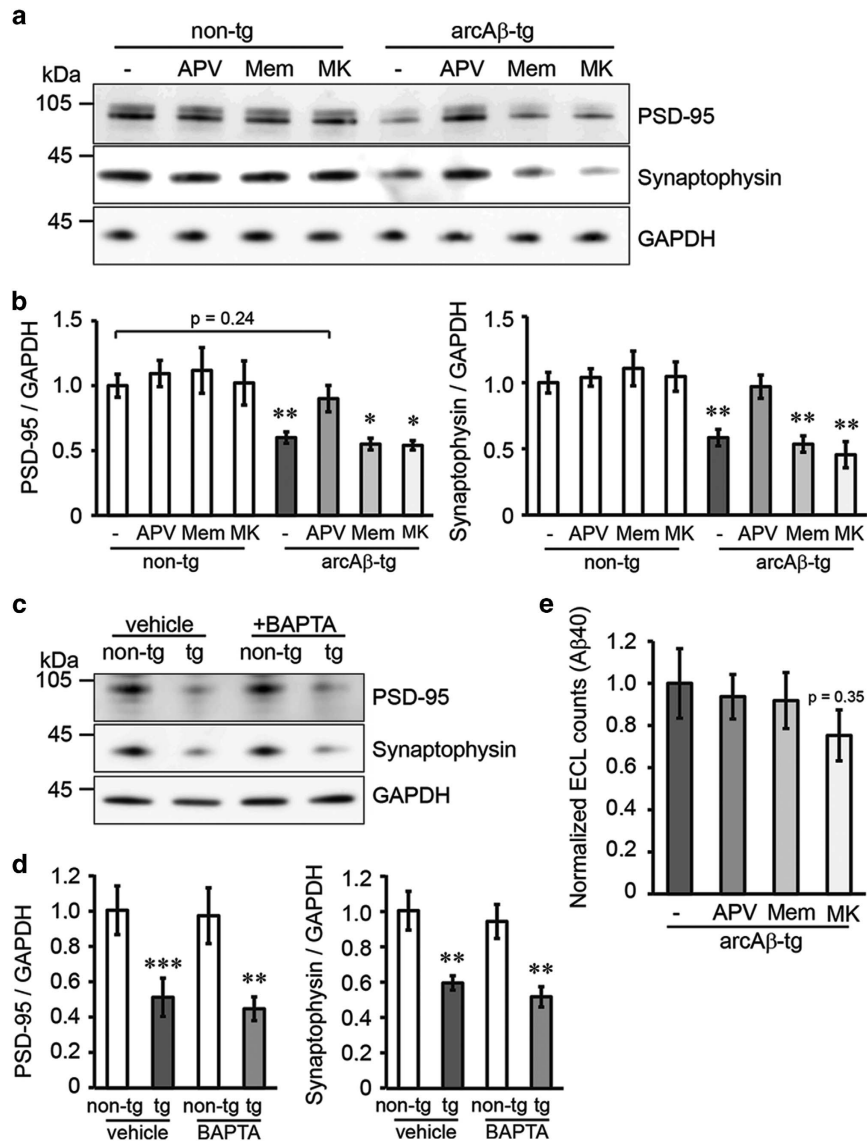


Figure 2 Blocking glutamate binding to NMDARs but not Ca²⁺ influx prevents the loss of pre- and postsynaptic markers in arcA β -transgenic cultures. (a) Representative western blot of cell lysates from non-transgenic or arcA β -transgenic cultures after treatment with NMDAR antagonists APV (100 μ M), memantine (1 μ M) or MK-801 (30 μ M). (b) Quantification of western blots. PSD-95 and synaptophysin levels are strongly reduced in arcA β -transgenic cultures. APV treatment restores PSD-95 and synaptophysin signals back to control levels, whereas memantine and MK-801 have no effect. $n=6$. (c) Representative western blot of cell lysates from non-transgenic or arcA β -transgenic slices after treatment with Ca²⁺ chelator BAPTA (2 mM) or vehicle (BAPTA solvent NaHCO₃). (d) BAPTA treatment does not affect loss of synaptic proteins in transgenic cultures. $n=6$. (e) A β 40 levels in the supernatant of arcA β -transgenic cultures after treatment with NMDAR antagonists measured by MSD. A β 40 production is not influenced by any NMDAR antagonist. A β levels were corrected by protein levels from lysates. $n=3$. All values are shown as mean \pm S.E.M. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; two-tailed unpaired Student's t -test; significances indicate differences to the respective non-transgenic control). non-tg, non-transgenic; tg, arcA β -transgenic; Mem, memantine; MK, MK-801

oligomer-induced spine loss. Oligomeric A β further reduced PSD-95 and synaptophysin levels, which could not be rescued by BAPTA treatment (Figures 4i and j). A β did not cause cell death at the used concentration (Figure 4l).

This indicates that oligomeric A β , similar to transgenically produced A β , exerts its toxic properties on synapses via NMDAR signaling, independent of Ca²⁺ influx.

To determine whether Ca²⁺ flux-independent synaptic loss depends on G protein signaling, we treated slices with oligomeric A β and pertussis toxin (PTX), an inhibitor of the heterotrimeric G_{i/o} protein family, at concentrations described

before in slice cultures.²⁶ PTX administration did not prevent spine loss caused by A β (Figures 5a and b) suggesting that A β -induced synaptic loss does not require a PTX-sensitive subgroup of G proteins.

Discussion

In this study, we have examined the role of Ca²⁺ flux for A β -induced loss of dendritic spines and pre- and postsynaptic proteins. Our data show that NMDAR-dependent ion flux is not required for synaptic loss, whereas binding of glutamate to the

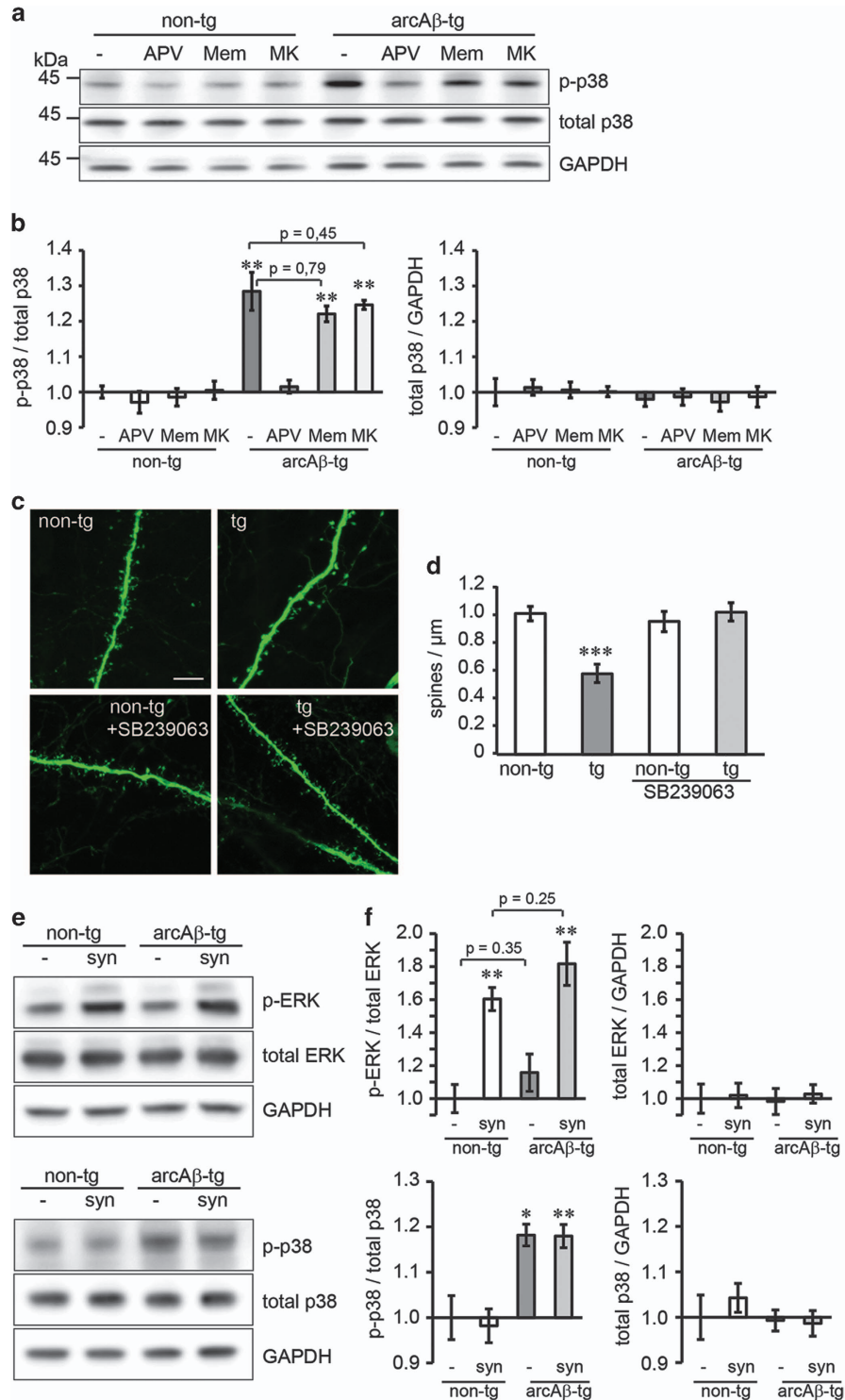
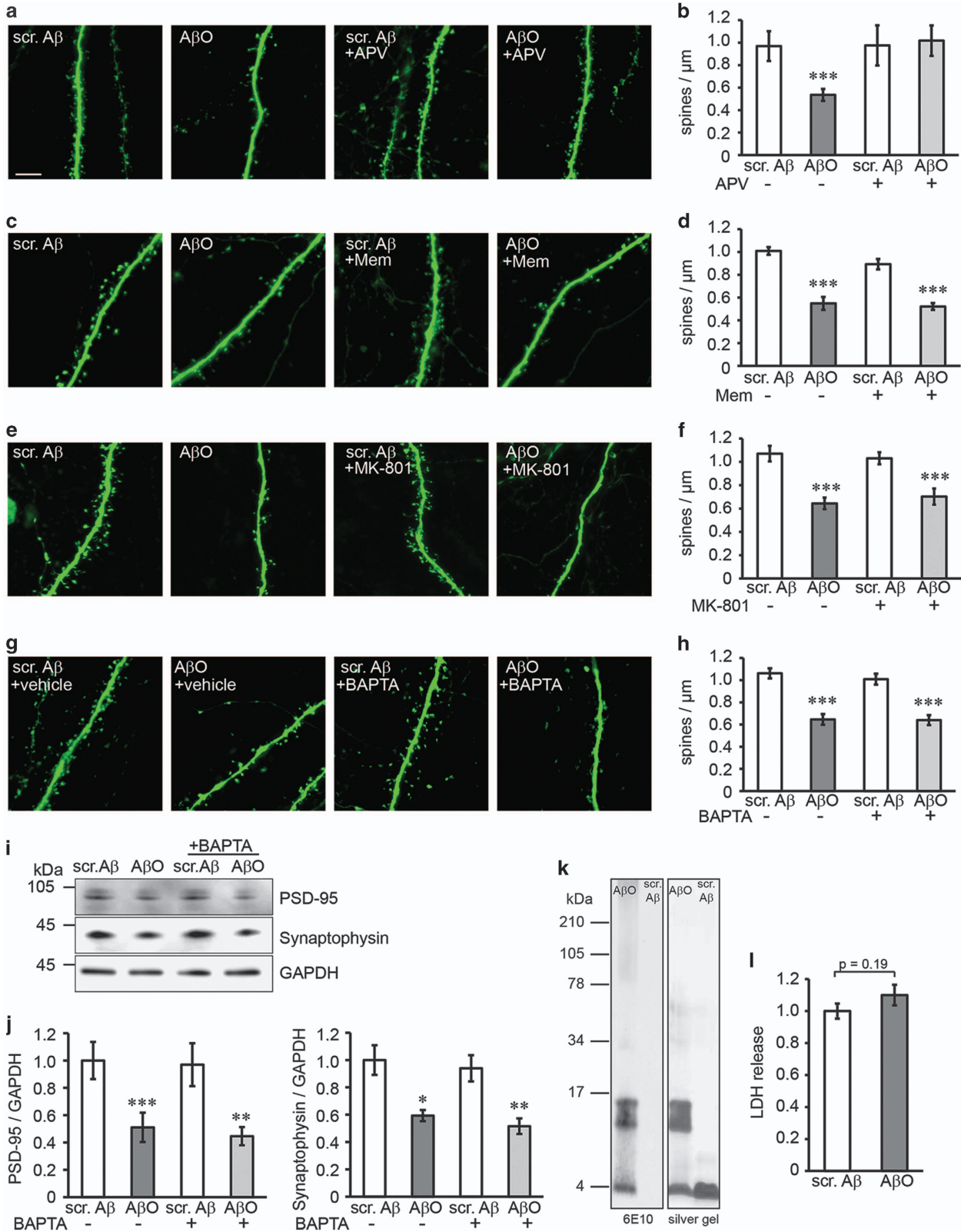


Figure 3 p38 MAPK is activated in arcA β -transgenic cultures and mediates spine loss. (a) Representative western blot showing phosphorylated (active) and total p38 MAPK in lysates non-transgenic or arcA β -transgenic slices after treatment with different NMDAR antagonists. (b) Quantification shows increased levels of phosphorylated p38 in arcA β -transgenic cultures. The increased amounts of phospho-p38 were reduced to control levels by APV (100 μ M) but not by memantine (1 μ M) or MK-801 (30 μ M) treatment. The non-transgenic untreated control was set to 1. $n=6$. (c) Confocal images of dendrites from CA1 neurons in the stratum radiatum of non-transgenic and arcA β -transgenic hippocampal slice cultures treated with p38 MAPK kinase inhibitor SB239063 (20 μ M). Scale bar: 5 μ m. (d) SB239063 treatment reverses the dendritic spine loss in arcA β -transgenic cultures. $n=14-16$. (e) Representative western blot showing activated ERK (p-ERK) and activated p38 (p-p38) in non-transgenic or arcA β -transgenic slices after synaptic activation with bicuculline and 4-aminopyridine. (f) Quantification shows increased p-ERK levels after synaptic activation, independent of transgenic background. Synaptic activation does not affect p-p38 levels. $n=5$. All values are shown as mean \pm S.E.M. (* $P<0.05$, ** $P<0.01$, *** $P<0.001$; two-tailed unpaired Student's t -test; significances indicate differences to the respective non-tg control; for p-ERK/total ERK significances indicate differences to the respective non-activated culture). non-tg, non-transgenic; tg, arcA β transgenic; Mem, memantine; MK, MK-801; p-ERK, phospho-ERK; p-p38, phospho-p38 MAPK; syn, synaptic activation



NMDAR is essential for coupling A β with synaptic degeneration.

NMDARs have been thought to signal exclusively ionotropic, regulating intracellular signaling via Ca²⁺ transmission. However, recent evidence indicates that NMDARs can signal metabotropically, that is, independent of ion flux. The group of Roberto Malinow showed that induction of NMDAR-LTD via activation of p38 MAPK is based on metabotropic signaling.¹³ Further, the induction of LTD by A β can occur in the absence of Ca²⁺ transmission.^{17,27} Together, these data suggest that glutamate binding to NMDARs may induce a conformational change that subsequently activates intracellular signaling cascades even in the absence of Ca²⁺ flux. This possibly does

not exclude an additional role of intracellular Ca²⁺, because the injection of Ca²⁺ chelators into neurons prevents LTD induction.^{13,28} In agreement, oligomeric A β has been shown to increase intracellular Ca²⁺ levels by mobilizing Ca²⁺ from the ER rather than promoting influx of Ca²⁺ from the extracellular space.²⁹

Our data show that the induction of specific metabotropic-like NMDAR signaling pathway by A β , which is not induced by general synaptic activation, causes downstream phosphorylation of p38 MAPK. Active p38 MAPK is key player in NMDAR- and mGluR-dependent LTD^{30,31} and mediates AMPAR endocytosis.³² A study by Yang *et al.*³³ described an intracellular pathway based on the co-activation of mGluR5 and NMDARs, also independent of Ca²⁺ flux. Further, mGluR5 has been implicated in mediating toxic effects of A β at synapses.^{34,35} Thus, a co-activation of mGluR5 and NMDARs may cause downstream activation of p38 MAPK followed by synaptic loss. However, treatment with the G protein inhibitor PTX did not prevent spine loss in our model, which renders the involvement of mGluRs unlikely.

In previous studies, we showed that caspase-3 and calcineurin are essential for the loss of spines by A β .^{5,6} Caspase-3 can be activated by p38 MAPK.^{36,37} Further, D'Amelio and colleagues reported caspase-3- and calcineurin-mediated synaptic dysfunction in APP-transgenic mice. Importantly, they observed that caspase-3-activated calcineurin by proteolytic cleavage in a Ca²⁺-independent manner, supporting our finding that A β -induced synaptic dysfunction can occur in the absence of Ca²⁺ flux.

An important finding in our study is the lack of synaptic protection by memantine as memantine is the only clinical approved NMDAR antagonist for treatment of AD patients. Memantine, at clinically relevant low μ M concentrations, is a low-affinity, uncompetitive open-channel blocker with a relatively high off-rate.³⁸ High-affinity NMDAR antagonists may be toxic after long exposure due to block of synaptic transmission. However, memantine has been suggested to be more tolerable because of blocking mainly over-excitation of the receptor rather than its physiological activity. As the high off-rate of memantine could still allow Ca²⁺ influx into the cell, we confirmed the data using a second open-channel blocker MK-801 and the Ca²⁺ chelator BAPTA. Further, all used compounds could fully block ERK phosphorylation after synaptic activation. Interestingly, treatment of slices with even the high-affinity inhibitors D-APV or MK-801 or with BAPTA did not show any side effects on spines.

Despite having no protective effect for A β -induced synaptic loss in our study, memantine may be more beneficial

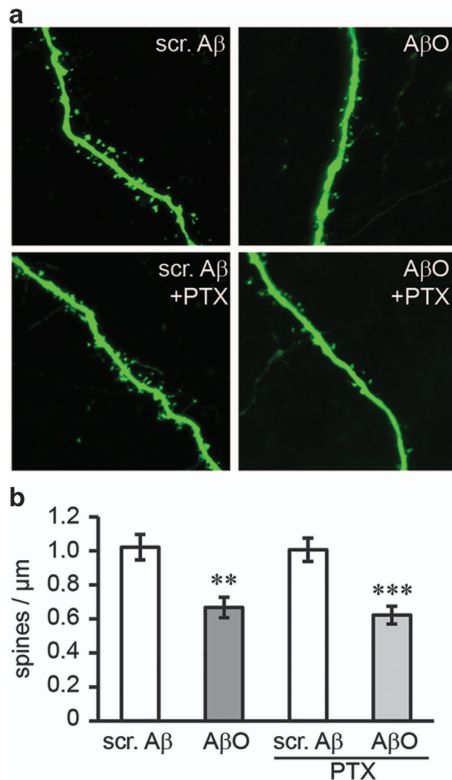


Figure 5 Oligomeric A β -induced synaptic loss is not prevented by treatment with PTX. (a) Confocal images of dendrites from CA1 neurons in the stratum radiatum of non-transgenic slice cultures treated with oligomeric A β (500 nM) or scrambled A β (500 nM) and G protein inhibitor PTX (500 ng/ml). (b) PTX treatment does not prevent A β oligomer-induced dendritic spine loss. $n = 12$. Values are shown as mean \pm S.E.M. (** $P < 0.01$, *** $P < 0.001$; two-tailed unpaired Student's t -test). Scr. A β , scrambled A β ; A β O, oligomeric A β ; PTX, pertussis toxin

Figure 4 Oligomeric A β -induced synaptic loss is prevented by APV but not by memantine, MK-801 or BAPTA treatment. (a) Confocal images of dendrites from CA1 neurons in the stratum radiatum of non-transgenic slice cultures treated with oligomeric A β (500 nM) or scrambled A β (500 nM) and NMDAR antagonist APV (100 μ M). Scale bar: 5 μ M. (b) APV treatment prevents A β oligomer-induced dendritic spine loss. $n = 13-17$. (c) Confocal images of non-transgenic cultures treated with oligomeric A β (500 nM) and NMDAR antagonist memantine (1 μ M). (d) Memantine treatment does not prevent spine loss. $n = 12-17$. (e) Confocal images of cultures treated with oligomeric A β (500 nM) and NMDAR antagonist MK-801 (30 μ M). (f) MK-801 treatment does not prevent spine loss. $n = 13-15$. (g) Non-transgenic cultures treated with A β oligomers (500 nM) and Ca²⁺ chelator BAPTA (2 mM) or vehicle (BAPTA solvent NaHCO₃). (h) BAPTA does not prevent spine loss caused by oligomeric A β . $n = 11-15$. (i) Representative western blot of cell lysates from slices after treatment with A β oligomers (500 nM) and Ca²⁺ chelator BAPTA (2 mM). (j) BAPTA does not prevent reduction in PSD-95 or synaptophysin levels after A β oligomer treatment (500 nM) compared with scrambled A β . $n = 6$. Values are shown as mean \pm S.E.M. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; two-tailed unpaired Student's t -test). Scr. A β , scrambled A β ; A β O, oligomeric A β ; Mem, memantine

with respect to other $\text{A}\beta$ effects. A recent study showed that injection of low-molecular-weight (LMW) oligomers into mice caused persistent memory impairment and synaptic loss, whereas injection of high-molecular-weight (HMW) oligomers resulted in neuronal oxidative stress and reversible cognitive deficits but no synaptic loss. Memantine treatment could rescue only the effects of HMW but not LMW $\text{A}\beta$ oligomers,³⁹ further indicating that memantine may not be beneficial with respect to $\text{A}\beta$ -induced synaptic alterations. However, memantine protected against the induction of oxidative stress by oligomeric $\text{A}\beta$ ¹⁰ and studies from our lab showed that memantine, at the concentration used in this study, prevented the increase in tau phosphorylation by $\text{A}\beta$ (unpublished observations).

Because synaptic loss occurs early in the disease process,⁴⁰ our data may contribute to explain why memantine is ineffective in treating early-staged mild AD patients.⁴¹ Together, our data establish a Ca^{2+} flux- and G protein-independent NMDAR signaling pathway coupling $\text{A}\beta$ toxicity with p38 MAPK activation and synapse loss, suggesting pharmacological inhibition of this pathway as a potent mechanism to prevent $\text{A}\beta$ -mediated early synaptic loss.

Materials and Methods

Chemicals/reagents. Cell culture reagents were purchased from Sigma (Schnelldorf, Germany) and Invitrogen (Basel, Switzerland). NMDA receptor antagonists D-APV (also called D-AP5, *D*-2-amino-5-phosphonovalerate; Batch No.:71), MK-801 ((5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine maleate; Batch No.:8), memantine (3,5-dimethyl-tricyclo[3.3.1.1^{3,7}]decan-1-amine hydrochloride; Batch No.:9), Ca^{2+} chelator BAPTA (Batch No.:4), AMPA receptor antagonist CNQX (6-cyano-7-nitroquinoxaline-2,3-dione; Batch No.:33) were purchased from Tocris (Bristol, UK). PTX was purchased from List Biological Laboratories (Campbell, CA, USA).

Hippocampal slice cultures. Arc $\text{A}\beta$ -transgenic mice were obtained as described.²³ All animal experiments were performed in accordance with the guidelines of the Swiss veterinary cantonal office. Hippocampal slice cultures were prepared and cultured as described.⁴² In short, 6–7-day-old transgenic and non-transgenic C57BL/6 mice were decapitated, brains were removed, hippocampi were isolated and cut into 400- μm thick slices. Slices were cultured in culture medium (minimum essential medium Eagle with HEPES modification, 25% basal medium with Earle's modification, 25% heat-inactivated horse serum, 2 mM glutamine, 50 units per ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin, 0.6% glucose, pH 7.2). Culture medium was exchanged every second or third day. On DIV 11, culture medium was replaced by low-serum Nb-N2 medium (Neurobasal medium, 0.5% heat-inactivated horse serum, 2 mM glutamine, 50 units per ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin, 0.6% glucose, 1 \times N2 supplement, pH 7.2) to ensure more defined condition during analysis. For spine analysis, slice cultures were infected with Sindbis virus expressing EGFP on DIV 12 in culture and fixed on DIV 15 with 4% paraformaldehyde/sucrose. For protein analysis, uninfected slices were lysed on DIV 15 in culture.

Treatments. To determine inhibitor effects in transgenic cultures, slices were treated with respective inhibitors from DIV 11–15. To analyze effects of oligomeric $\text{A}\beta$, slices were treated with $\text{A}\beta$ oligomers or scrambled $\text{A}\beta$ from DIV 11–15. To assess the effects of inhibitors on cultures exposed to oligomeric $\text{A}\beta$, slices were treated with $\text{A}\beta$ oligomers and the respective inhibitor in parallel from DIV 11–15. For treatment with PTX, slices were exposed to PTX from DIV 13–15.

Dendritic spine analysis. To determine dendritic spine density, virus solution was diluted to achieve 1–10 infected neurons per slice to allow imaging of single dendritic fragments. Analysis of dendritic spine density was performed using Leica SP2 CLSM equipped with 63 \times objective (NA: 1.2) and 488-nm Argon laser. Apical dendritic segments in CA1 stratum radiatum were imaged with size of 30 \times 30 μm

(512 \times 512 pixel, voxel size: 0.05813 \times 0.05813 \times 0.25 μm). Image stacks were processed to maximum projections, and dendritic spine density was determined using ImageJ.

Spine imaging and counting were performed blinded (without the researcher knowing the mouse genotype or culture treatment).

Synaptic activation protocol. Stimulation of synaptic activity was adapted from Tackenberg *et al.*⁶ Cultures were pretreated with APV, memantine, MK-801 or BAPTA for 12 h before activation. Then, cultures were exposed to neurobasal medium containing 1 mM 4-AP, 25 mM bicuculline and the respective inhibitor for 20 min. Control cultures were treated with neurobasal medium containing identical DMSO concentrations as above but devoid of 4-AP, bicuculline and inhibitors.

Western blot. Cultured slices were harvested on DIV 15, sonicated in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.5% deoxycholate and 0.1% SDS, pH 8.0) containing phosphatase inhibitor cocktails 1 and 2 (Sigma) and protease inhibitor cocktail (Roche, Basel, Switzerland) and centrifuged at 5000 g for 10 min at 4 $^{\circ}\text{C}$. The supernatant was stored at -80°C . Lysates were subjected to SDS-PAGE followed by immunoblotting using primary antibodies against PSD-95 and Synaptophysin (both Millipore, Billerica, MA, USA, 1 : 1000), phospho-p38 MAPK and p38 MAPK (both Cell Signaling, Danvers, MA, USA, 1 : 1000), 6E10 (Signet, Dedham, MA, USA, 1 : 500) and GAPDH (Biodesign, Saco, ME, USA, 1 : 5000). Band intensities were quantified with ImageJ.

Silver staining. Oligomeric $\text{A}\beta$ 42 preparations were analyzed by SDS-PAGE. The gel was left overnight in fixing solution (40% EtOH, 10% acetic acid), sensitized in 0.017% sodium thiosulfate for 2 min, impregnated in 0.27% silver nitrate solution (including 0.37% formaldehyde) for 30 min and developed in 0.03 M sodium carbonate (supplemented with 0.15% formaldehyde and 0.02% sodium thiosulfate). The reaction was stopped in 3% glacial acid.

$\text{A}\beta$ oligomer preparations. Synthetic $\text{A}\beta$ 42 peptide was obtained from American peptide. Preparation of $\text{A}\beta$ 42 oligomers (Abeta-derived diffusible ligands) was carried out as previously described.⁴³ Cold 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) was added to $\text{A}\beta$ 42 peptide to a final concentration of 1 mM. HFIP was evaporated overnight, peptides dried for 10 min in a speedvac and stored at -80°C . Peptides were resuspended in DMSO at 5 mM concentrations. Neurobasal medium without phenol red was added to achieve a peptide concentration of 100 μM and incubated for 24 h at 4 $^{\circ}\text{C}$. Higher aggregates, for example, fibrils were removed by centrifugation at 14 000 g for 10 minutes at 4 $^{\circ}\text{C}$ and the supernatant was used for experimental procedures. $\text{A}\beta$ 42 oligomer preparations were analyzed by silver staining and western blot for each experiment.

Conflict of Interest

The authors declare no conflict of interest.

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