

Inhibition of the Motor Protein Eg5/Kinesin-5 in Amyloid β -Mediated Impairment of Hippocampal Long-Term Potentiation and Dendritic Spine Loss

Ronald K. Freund, Emily S. Gibson, Huntington Potter, and Mark L. Dell'Acqua

Department of Pharmacology (R.K.F., E.S.G., M.L.D.'A.), and Department Neurology (H.P.), School of Medicine, and Linda Crnic Institute for Down Syndrome (M.L.D.'A., H.P.), Anschutz Medical Campus, University of Colorado, Aurora, Colorado

Received December 18, 2015; accepted March 7, 2016

ABSTRACT

Alzheimer's disease (AD) is characterized by neurofibrillary tangles, amyloid plaques, and neurodegeneration. However, this pathology is preceded by increased soluble amyloid beta ($A\beta$) 1–42 oligomers that interfere with the glutamatergic synaptic plasticity required for learning and memory, including *N*-methyl-D-aspartate receptor (NMDAR)-dependent long-term potentiation (LTP). In particular, soluble $A\beta$ (1–42) acutely inhibits LTP and chronically causes synapse loss. Many mechanisms have been proposed for $A\beta$ -induced synaptic dysfunction, but we recently found that $A\beta$ (1–42) inhibits the microtubule motor protein Eg5/kinesin-5. Here we compared the impacts of $A\beta$ (1–42) and monastrol, a small-molecule Eg5 inhibitor, on LTP in hippocampal slices and synapse loss in neuronal cultures. Acute (20-minute) treatment with monastrol, like $A\beta$, completely inhibited LTP at doses >100 nM. In addition, 1 nM $A\beta$ (1–42) or 50 nM monastrol inhibited LTP

~50%, and when applied together caused complete LTP inhibition. At concentrations that impaired LTP, neither $A\beta$ (1–42) nor monastrol inhibited NMDAR synaptic responses until ~60 minutes, when only ~25% inhibition was seen for monastrol, indicating that NMDAR inhibition was not responsible for LTP inhibition by either agent when applied for only 20 minutes. Finally, 48 hours of treatment with either 0.5–1.0 μ M $A\beta$ (1–42) or 1–5 μ M monastrol reduced the dendritic spine/synapse density in hippocampal cultures up to a maximum of ~40%, and when applied together at maximal concentrations, no additional spine loss resulted. Thus, monastrol can mimic and in some cases occlude the impact of $A\beta$ on LTP and synapse loss, suggesting that $A\beta$ induces acute and chronic synaptic dysfunction in part through inhibiting Eg5.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease that affects cognition primarily in the elderly. With advances in modern medicine successfully treating other diseases and leading to overall increased life spans, it is estimated that the number of people with AD will likely exceed 15 million in the United States and 50 million worldwide by 2050. However, there are currently no effective treatments for AD, so it is expected that the already substantial burdens that caring for AD patients places on the U.S. and world economies and health care systems will increase dramatically in the future (Wimo and Prince, 2011).

The hallmarks of AD include amyloid plaques and neurofibrillary tangles found in the postmortem brain. Plaques contain insoluble filaments of amyloid beta ($A\beta$) peptides,

which arise from proteolytic processing of the amyloid precursor protein (APP), a transmembrane cell-adhesion protein. Similarly, tangles are oligomers of hyperphosphorylated tau, a microtubule-associated protein. However, plaques and to a lesser extent tangles are poor indicators of cognitive impairment in AD, especially in early disease stages before extensive tangle formation and plaque deposition (Terry et al., 1991; Dickson et al., 1995). Instead, soluble forms of $A\beta$ are thought to be largely responsible for driving brain dysfunction in early AD (McLean et al., 1999; Naslund et al., 2000) by interfering with excitatory synaptic function through a variety of mechanisms, including by promoting tau phosphorylation (reviewed in Mucke and Selkoe, 2012).

Accordingly, acute application of synthetic $A\beta$ (1–42) peptides (Lambert et al., 1998) or $A\beta$ extracted from AD brains (Shankar et al., 2007, 2008) can disrupt synaptic plasticity in brain regions that are important for learning and memory, such as inhibiting long-term potentiation (LTP) at hippocampal cornu ammonis 1 (CA1) synapses. Although $A\beta$ (1–42) monomers do not alter LTP, $A\beta$ forms low-molecular-weight, soluble oligomers, including dimers and trimers, that potently inhibit LTP (McLean et al., 1999; Naslund et al., 2000; Walsh

This work was supported by the National Institutes of Health National Institute of Neurological Disorders and Stroke [Grant NS076291 (to H.P.) and Grant# NS040701 (to M.L.D.)] and the Linda Crnic Institute for Down Syndrome (to H.P. and M.L.D.). The contents are the authors' sole responsibility and do not necessarily represent official National Institutes of Health views.

dx.doi.org/10.1124/mol.115.103085.

ABBREVIATIONS: $A\beta$, amyloid beta; aCSF, artificial cerebrospinal fluid; AD, Alzheimer's disease; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMPAR, AMPA glutamate receptor; APP, amyloid precursor protein; APV, (DL)-amino-5-phosphonovaleric acid; CA1, cornu ammonis 1; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DIV, day in vitro; fEPSP, field excitatory postsynaptic potential; GFP, green fluorescent protein; HFS, high-frequency stimulation; LTP, long-term potentiation; NMDAR, *N*-methyl-D-aspartate receptor.

et al., 2002; Shankar et al., 2007, 2008; Selkoe, 2008). Several hypotheses have been posited to explain how A β inhibits LTP and alters brain function through perturbing various signaling pathways. In this regard A β can interact with a number of different membrane receptors, including *N*-methyl-D-aspartate receptors (NMDAR) (Shankar et al., 2007; Kurup et al., 2010; Ari et al., 2014), metabotropic glutamate receptors (Hsieh et al., 2006; Um et al., 2013), α 7 nicotinic acetylcholine receptors (Wang et al., 2000a,b; Snyder et al., 2005; Liu et al., 2012), paired immunoglobulin-like receptor B (Kim et al., 2013), cellular prion protein (Lauren et al., 2009; Um et al., 2013), and amylin receptors (Kimura et al., 2012).

However, A β is not only extracellular but also accumulates intracellularly where it may interact with additional targets relevant for LTP inhibition, synapse loss, and cognitive impairment. Furthermore, exogenously added A β can enter cells from the medium. Indeed, we recently found that A β (1–42) inhibits the activity of the microtubule-dependent motor protein Eg5/kinesin-5 (also known as KIF11), which regulates a number of different important microtubule-dependent functions in neuronal and non-neuronal cells, including chromosome segregation, growth cone turning, and microtubule organization in axons and dendrites that may also indirectly impact membrane trafficking (Baas, 1998; Ferhat et al., 1998; Nadar et al., 2008; Borysov et al., 2011; Ari et al., 2014; Kahn et al., 2015).

LTP is induced by Ca²⁺ influx through NMDARs and is expressed by increased function of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors (AMPA) that are recruited to the synapse via membrane trafficking. Alterations in membrane trafficking of NMDARs and AMPARs as well as phosphorylation of microtubule regulatory proteins such as tau are thought to be particularly relevant for A β -mediated synaptic dysfunction (reviewed in Mucke and Selkoe, 2012). Thus, we further investigated possible roles for Eg5 inhibition in A β (1–42)-mediated synaptic dysfunction by exploring the similarity of actions of monastrol, a specific small-molecule inhibitor of Eg5 (Maliga et al., 2002; Cochran et al., 2005), with those of A β (1–42) on acute inhibition of LTP in hippocampal slices and on chronic promotion of dendritic spine/synapse loss in neuronal cultures. Importantly, our findings are consistent with A β promoting synaptic dysfunction at least in part through inhibition of Eg5/kinesin-5.

Materials and Methods

Animals

Experiments were performed on C57/BL6 male mice, 14 to 21 days old. All animal procedures were conducted in accordance with National Institutes of Health/Public Health Service guidelines and with the approval of the Denver Institutional Animal Care and Use Committee of the University of Colorado.

Pharmacologic Reagents

All chemical were obtained from Sigma-Aldrich (St. Louis, MO) except where noted otherwise. We obtained 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and (DL)-amino-5-phosphonovaleric acid (APV) from Tocris Bioscience/R&D Systems (Minneapolis, MN). Monastrol was obtained from Sigma-Aldrich. Synthetic A β (1–42) peptide was obtained from AnaSpec (Fremont, CA). The stock solutions were prepared as follows.

Method 1. For electrophysiology experiments, 1 mg A β (1–42) was dissolved in 80 μ l NH₄OH (1 M) + 120 μ l dimethylsulfoxide (final concentration 1.1 mM), aliquoted, flash frozen, and stored at -80° C until use. Aliquots were then thawed on the day of use and diluted to the appropriate working concentration(s) in artificial cerebrospinal fluid (aCSF).

Method 2. For dendritic spine loss experiments, 1 mg A β (1–42) was dissolved in 440 μ l of 1,1,1,3,3,3-hexafluoro-2-propanol, aliquoted per 100 μ g; the 1,1,1,3,3,3-hexafluoro-2-propanol evaporated under nitrogen, and then the dried A β (1–42) peptide was stored at -80° C until use.

On the day before use, a single A β (1–42) peptide aliquot was dissolved in 2.2 μ l of dimethylsulfoxide for 1 hour at room temperature, diluted with phosphate-buffered saline to a concentration of 100 μ M, stored for 24 hours at 4 $^{\circ}$ C, and centrifuged at 14,000g for 10 minutes. The supernatant was then removed and diluted into the neuronal culture medium to achieve the desired final working concentration(s).

Using either of these methods, the resulting solutions contained predominantly A β (1–42) monomers and low-molecular-weight soluble oligomers (dimers and trimers), with no detectable presence of higher molecular weight species, as shown in Fig. 1 by electrophoresis under nonreducing conditions on 10% to 20% Tris-Tricine gels (BioRad Laboratories, Hercules, CA), transfer to 0.2 μ m reinforced nitrocellulose (Whatman/GE Healthcare, Maidstone, Kent, United Kingdom), and immunoblotting with anti-A β antibodies (1:10,000; Covance, Princeton, NJ).

Electrophysiology

Mouse Hippocampal Slice Preparation. After the mice had been sacrificed, their brains were rapidly removed and immersed in ice-cold sucrose containing cutting buffer (in mM: 2 KCl, 12 MgCl₂, 0.2 CaCl₂, 1.25 NaH₂PO₄, 10 D-glucose, 220 sucrose, and 26 NaHCO₃) for 40 to 60 seconds to cool the interior of the brain. Transverse slices (400 μ m thickness) were made using a McIlwain tissue chopper/slicer.

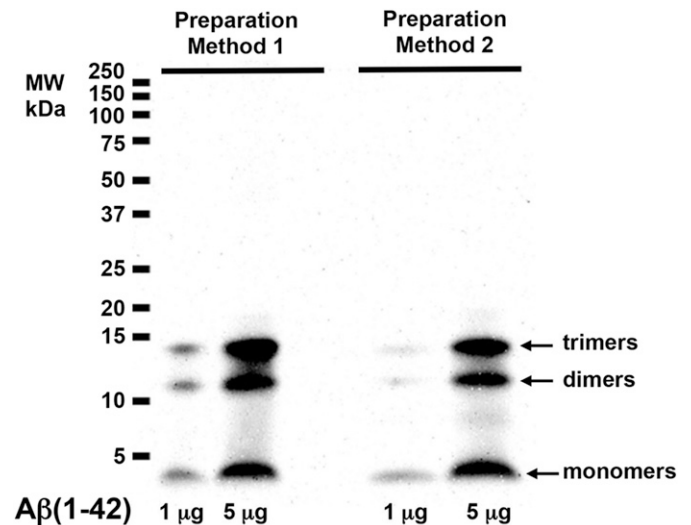


Fig. 1. Preparation of soluble, low-molecular-weight A β (1–42) oligomers. Nondenaturing 10%–20% Tris-Tricine gel electrophoresis and immunoblotting with anti-A β antibodies demonstrates successful preparation of solutions containing predominantly A β (1–42) monomers and low-molecular-weight, soluble oligomers (dimers and trimers), with no detectable presence of higher molecular weight species. Both preparation method 1, used for slice electrophysiology experiments, and method 2, used for neuronal culture spine loss experiments, produce similar results. *Note:* Under nonreducing conditions it is difficult to make accurate molecular weight determinations based on relative migration compared with molecular weight standards.

The slices were transferred to individual compartments in a storage system for at least 60 minutes. After recovery, a single slice was transferred to a recording chamber and superfused with aCSF at a bulk flow rate of 2–3 ml/min at 31°C. The aCSF contained the following (in mM): 124 NaCl, 3.5 KCl, 1.3 MgCl₂, 2.5 CaCl₂, 1.2 NaH₂PO₄, 11 D-glucose, 25.9 NaHCO₃, and 0.0025 picrotoxin (Tocris Bioscience).

Baseline Recordings. Before each slice recording experiment, an input–output curve was generated by increasing the stimulus voltage and recording the synaptic response until either a maximum was reached or evidence of a population spike was observed on the field excitatory postsynaptic potential (fEPSP) response.

LTP Measurements. Synaptic fEPSP responses were evoked with bipolar tungsten electrodes placed in Schaffer collateral axon pathway to the CA1 dendritic field layer. Test stimuli were delivered once every 20 seconds with the stimulus intensity set to 40% to 50% of the maximum synaptic response. High-frequency stimulation (HFS) consisted of two trains of 100 Hz stimuli lasting 1 second each, with an intertrain interval of 20 seconds, at the control stimulus intensity. The fEPSP recordings were made with a glass micropipette filled with aCSF and placed in the stratum radiatum approximately 200–300 μm from the cell body layer. This stimulation produced LTP that persisted for more than 60 minutes in wild-type animals.

The initial slopes of fEPSPs were calculated as the slope measured between 10% and 30% from the origin of the initial negative deflection. Each time point shown is an average of six 20-second interval measurements. An average over 10 minutes of LTP recordings 55 to 65 minutes after HFS was used in the summary analyses.

Neuronal Cultures

Hippocampal neuron cultures were prepared from postnatal day 0–2 C57/BL6 male and female mice, plated at medium density (300–450 cells/mm²) on glass coverslips and maintained in Neurobasal plus B27 (Invitrogen) and GlutaMAX (Invitrogen) until transfection with a plasmid encoding green fluorescent protein (GFP)(pEGFPN1; Clontech, Mountain View, CA) using Lipofectamine 2000 (Invitrogen) on day in vitro (DIV) 11–12 as previously described (Robertson et al., 2009; Keith et al., 2012). After 1 day of GFP expression on DIV 12–13, the cultures were then left untreated (controls) or treated for two additional days with Aβ(1–42) and/or monastrol added to the culture media. On DIV 14–15 after treatment, neurons were fixed in 3.7% formaldehyde and then the coverslips mounted on slides with Pro-Long Gold (Invitrogen). Images of GFP transfected dendrites were then acquired on an Axiovert 200M microscope (Zeiss, Thornwood, NY) with a 63x objective (1.4NA, plan-Apo) and a CoolSNAP2 (Photometrics, Tucson, AZ) CCD camera. Focal plane z-stacks (0.5 μm spacing) were acquired, deconvolved to correct for out of focus light, and 2D maximum intensity projections generated (Slidebook 5.0–6.0, Intelligent Imaging Innovations). Spine numbers were quantified from projection images using the ruler tool in Slidebook 5.0–6.0 software with manual counting as in Robertson et al., 2009 and expressed as the number of spines / 10 μm of dendrite using measurements obtained for multiple lengths of dendrite (N = number of lengths of dendrite) taken from multiple images across three independent neuronal cultures for each experimental treatment condition.

Statistical Methods

Group comparisons to control were performed in Prism (GraphPad Software, San Diego, CA) using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc analysis, in which case only $P > 0.05$ (not statistically significant), $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ are provided by the software. Pairwise comparisons were performed in Prism or Excel (Microsoft, Redmond, WA) using Student's *t* test. In all cases, $P < 0.05$ was considered statistically significant, and data are expressed as mean ± S.E.M. error bars.

Results

Many previous studies have shown that acute application of Aβ(1–42) can inhibit LTP at hippocampal CA1 synapses. Accordingly, we confirmed that 20 minutes of preincubation of hippocampal slices with 100 nM Aβ(1–42) strongly inhibited LTP of the AMPAR synaptic response measured as the slope of the fEPSP by extracellular recording at CA1 synapses (Fig. 2, A and B). We further investigated whether disabling one of the

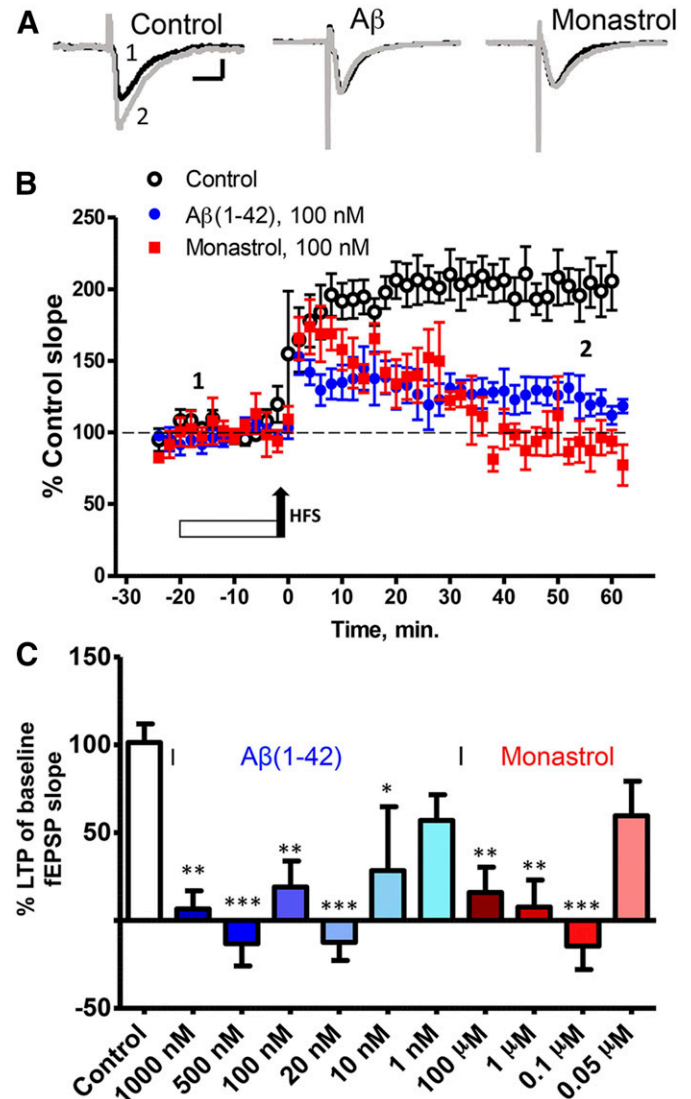


Fig. 2. Inhibition of early phase (up to 1 hour) of LTP by Aβ(1–42) and monastrol. (A) Typical fEPSPs before (black lines) and 60 minutes after (gray lines) HFS. (B) Time course of slope of fEPSPs before and after two HFS trains (black arrow: 1 × 100 Hz, 20 seconds apart). Open bar indicates the time of drug application. Aβ(1–42) (100 nM) or monastrol (100 nM) were applied to the bath 20 minutes before and including HFS, and the slopes and amplitudes of fEPSPs were recorded for 60 minutes after HFS. Numbers (1, 2) on time course indicate times when example signals in panel A were obtained. Data represent the mean ± S.E.M. for five slices from four animals for Aβ(1–42) and for monastrol. (C) LTP as a percentage of baseline slope (average of 50 to 60 minutes) for control (open bar) and various concentrations of Aβ(1–42) (blue bars) and monastrol (red bars). Data represent the mean ± S.E.M. for four to five slices from three to five mice for each concentration. Statistical significance from control LTP was determined by one-way analysis of variance with Dunnett's post hoc analysis. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

intracellular, cytosolic targets of $\text{A}\beta(1-42)$, the microtubule-dependent motor protein Eg5/kinesin-5, might also block LTP. Importantly, the 20-minute preincubation with 100 nM monastrol, a specific Eg5 inhibitor, also strongly inhibited LTP (Fig. 2, A and B). Additional dose–response analyses demonstrated that a wide range of concentrations of $\text{A}\beta(1-42)$ from 20 nM to 1 μM and monastrol from 100 nM to 100 μM caused essentially complete inhibition of LTP. However, slightly lower concentrations of 1 nM $\text{A}\beta(1-42)$ and 50 nM monastrol only partially inhibited LTP, with each causing a nonsignificant trend toward approximately 50% suppression (Fig. 2C).

Next we attempted to learn whether $\text{A}\beta(1-42)$ may act through the same mechanism(s) as monastrol, that is, by inhibiting Eg5. Thus, we applied each compound together at the concentrations that produced nonsignificant, partial LTP inhibition when tested alone to determine whether there would be any additional inhibition when coapplied. Importantly, when 1 nM $\text{A}\beta(1-42)$ and 50 nM monastrol were applied together, LTP was completely inhibited (Fig. 3, A and B). This enhanced inhibition suggests that $\text{A}\beta(1-42)$ and monastrol may each partially inhibit the same mechanism(s) required for LTP such that when added together more complete inhibition of LTP results. However, from these pharmacologic results alone, we cannot rule out that these two agents may also inhibit LTP in part through separate mechanisms.

$\text{A}\beta(1-42)$ and/or monastrol could inhibit LTP through altering the NMDAR activity that is necessary for LTP induction or by eventually suppressing the AMPAR activity that is required for maintenance of LTP expression. We investigated the first possibility by studying the NMDAR component of the fEPSP pharmacologically isolated by using 10 μM CNQX to block AMPARs and increasing the stimulus intensity. A test concentration of 100 nM $\text{A}\beta(1-42)$, which is higher than required to completely inhibit LTP (Fig. 2C), when applied for 60 minutes did not alter either the slope or amplitude of these isolated NMDAR synaptic responses (Fig. 4A). In contrast, application of 200 nM monastrol, which is also higher than that required to completely inhibit LTP (Fig. 2C), did cause a gradual and slight but persistent reduction (~20%–25%) in NMDAR responses, but this reduction only became statistically significant after 60 minutes of treatment (** $P < 0.001$; at end of drug application and persisting after washout; Fig. 4B). Importantly, no statistically significant inhibition of NMDAR activity was observed after only 20 minutes of treatment with monastrol (i.e., at the time when HFS was delivered to induce LTP in Figs. 2 and 3). Thus, NMDAR inhibition was not responsible for acute LTP inhibition by either $\text{A}\beta(1-42)$ or monastrol in our experiments. Likewise, neither 100 nM $\text{A}\beta(1-42)$ (Fig. 4C) nor 200 nM monastrol (Fig. 4D) resulted in any inhibition of the slope or amplitude of AMPAR fEPSP responses over 60 minutes of application and after washout for 30 minutes. Thus, direct inhibition of AMPAR transmission cannot account for the inhibitory actions of $\text{A}\beta(1-42)$ or monastrol on LTP.

Although we did not observe any decreases in synaptic AMPAR or NMDAR responses with only 1 hour of exposure to $\text{A}\beta(1-42)$, previous studies have found that more prolonged exposure to higher amounts of $\text{A}\beta(1-42)$ can substantially reduce synaptic NMDAR and AMPAR currents and also trigger synapse loss over a period of several days (Hsieh

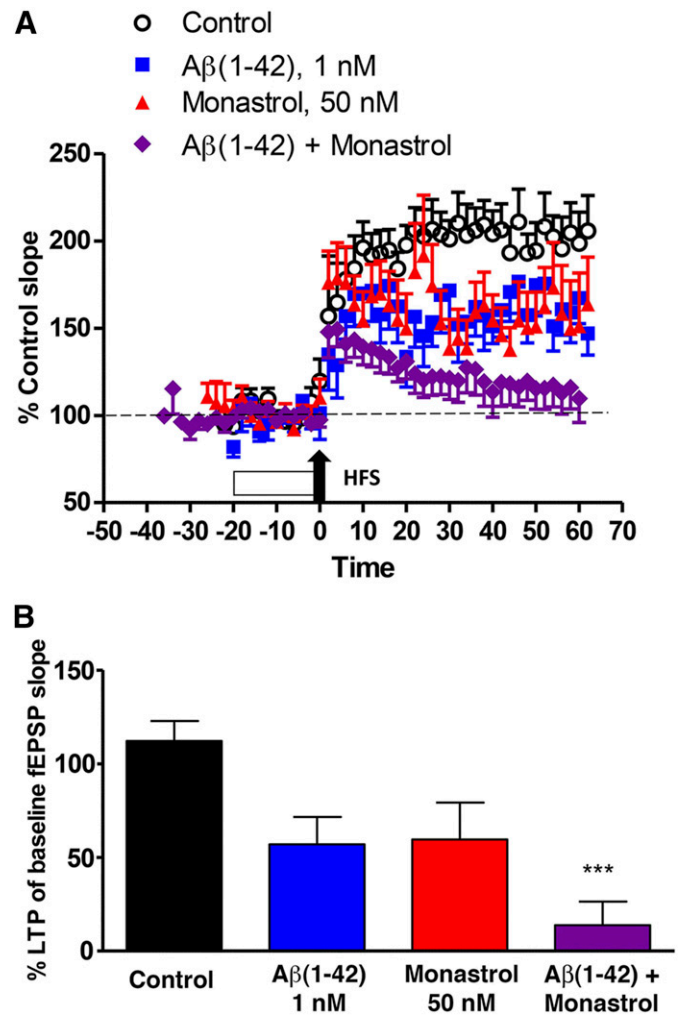


Fig. 3. Inhibition of LTP by lower concentrations of $\text{A}\beta(1-42)$, monastrol, and $\text{A}\beta(1-42)$ plus monastrol. (A) Time course for control (open black circles, $n = 9$; from same experiments as in Fig. 2B), 1 nM $\text{A}\beta(1-42)$ (solid blue squares, $n = 5$; from same experiments as in Fig. 2C), 50 nM monastrol (solid red triangles, $n = 8$; from same experiments as in Fig. 2C), and 1 nM $\text{A}\beta(1-42)$ plus 50 nM monastrol (solid purple diamonds, $n = 9$). Drugs were applied 20 minutes before and during HFS (100 Hz, 1 second, two trains 20 seconds apart). Data are the mean percentage potentiation \pm S.E.M. (B) Bar graphs depicting the mean percentage potentiation \pm S.E.M. over control during last 10 minutes of the recording period. At these concentrations, neither $\text{A}\beta(1-42)$ nor monastrol alone resulted in significant reduction of LTP, but when added together there was a statistically significant, complete inhibition of LTP (one-way analysis of variance with Dunnett's post hoc; *** $P < 0.001$).

et al., 2006; Shankar et al., 2007). In addition, prolonged exposure to $\text{A}\beta(1-42)$ or monastrol can also lead to reduced surface expression of AMPAR and NMDAR subunits (Snyder et al., 2005; Ari et al., 2014), perhaps due to both enhanced endocytosis and impaired delivery to the plasma membrane. Given that we observed a small reduction in synaptic NMDAR activity after 1 hour of Eg5 inhibition with monastrol, we next examined the impacts of longer $\text{A}\beta(1-42)$ and monastrol treatments on the number of dendritic spines in cultured mouse hippocampal neurons as an indicator of synapse loss (Fig. 5).

In agreement with many previous studies showing $\text{A}\beta(1-42)$ -induced synapse loss, application of 0.25–1 μM $\text{A}\beta(1-42)$ for 48 hours caused a concentration-dependent reduction in the number of spines (up to ~35% maximum loss; Fig. 5, A and B).

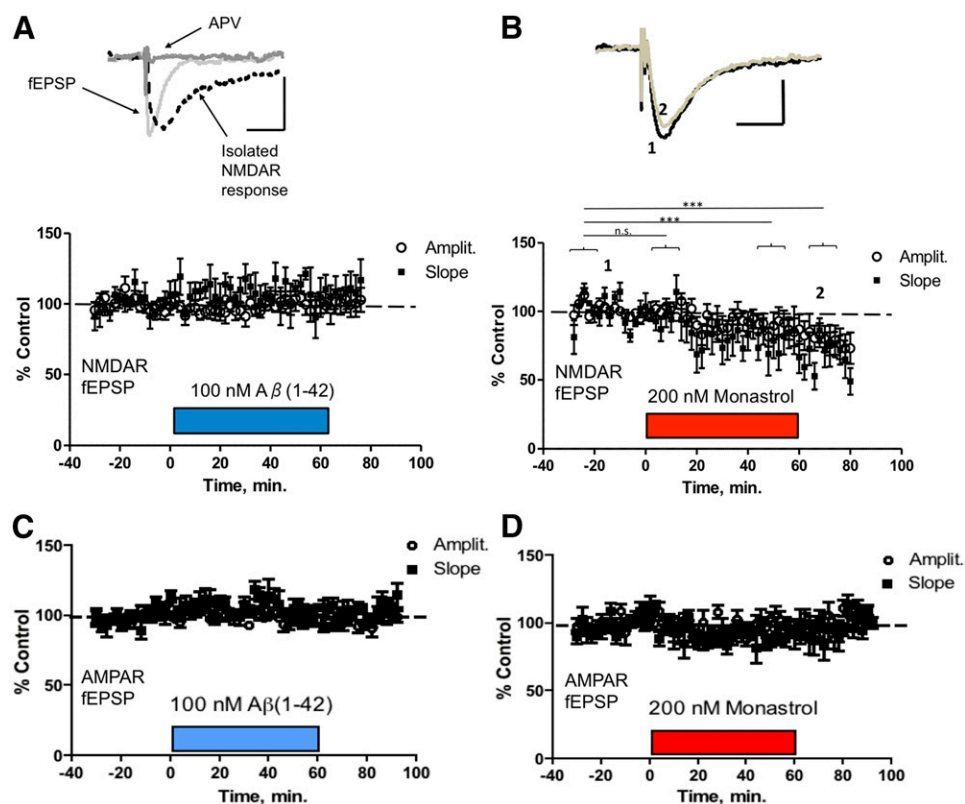


Fig. 4. Time course of $A\beta(1-42)$ and monastrol impacts on baseline NMDAR and AMPAR field potentials. (A) Upper panel depicts a representative fEPSP response before (light gray) and after (dotted black) pharmacologic isolation of the NMDAR component of the response by blocking the AMPAR component of the response with $10\ \mu\text{M}$ CNQX. Traces are normalized to the peak of the response to better show the distinct, slower kinetics of the isolated NMDAR component of the fEPSP. The isolated NMDAR fEPSP response is completely inhibited with $100\ \mu\text{M}$ of the antagonist DL-APV (gray). Lower panel: Time course of amplitudes and slopes of isolated NMDAR fEPSPs during application of $100\ \text{nM}$ $A\beta(1-42)$ ($n = 8$). (B) Upper panel: Representative isolated NMDAR fEPSPs before (1, black) and ~ 60 minutes after (2, light gray) application of monastrol ($n = 6$). Lower panel: Time course of amplitudes and slopes of isolated NMDAR fEPSPs before, during, and after application of $100\ \text{nM}$ monastrol ($n = 6$). One-way analysis of variance followed by Dunnett's post hoc analysis depicts no statistically significant change after 20-minute application of monastrol, but statistically significant decreases ($***P < 0.001$) in amplitude and slope at 60 and 90 minutes (the latter after a 30-minute wash). Calibration bars in upper panels: 1 mV, 20 milliseconds. (C) Time course of amplitudes and slopes of AMPAR fEPSPs during application of $100\ \text{nM}$ $A\beta(1-42)$ ($n = 5$). (D) Time course of amplitudes and slopes of AMPAR fEPSPs during application of $200\ \text{nM}$ monastrol ($n = 6$).

Similarly, monastrol ($0.5\text{--}5\ \mu\text{M}$) treatment also resulted in comparable spine loss ($\sim 40\%$ maximum loss). We then examined the effect of adding both $A\beta(1-42)$ and monastrol together on spine loss. Treatment with $0.5\ \mu\text{M}$ $A\beta(1-42)$ in addition to $5\ \mu\text{M}$ monastrol also resulted in $\sim 40\%$ loss of spines (Fig. 4), which was approximately the same degree of spine loss observed for each compound alone. Thus, the spine loss promoted by monastrol occluded any additional spine loss by $A\beta(1-42)$, a result that is consistent with shared/overlapping mechanisms for reduction in spine density by $A\beta(1-42)$ and monastrol in hippocampal neurons.

Discussion

AD is a neurodegenerative disorder that disrupts memory and cognitive abilities. The underlying molecular mechanisms of AD are diverse, but one of the primary lines of current research is that soluble oligomeric forms of $A\beta(1-42)$ and $A\beta(1-40)$ accumulate in and around excitatory synapses to alter normal processes of learning long before the appearance of amyloid plaques and neuronal cell loss. In particular, many studies have shown that $A\beta(1-42)$ blocks LTP (Chen et al., 2000; Walsh et al., 2002; Selkoe, 2008), a cellular mechanism thought to underlie memory acquisition. The data presented

here confirm that a range of $A\beta(1-42)$ concentrations applied to hippocampal slices in vitro inhibit LTP at Schaffer collateral to CA1 synapses.

A number of mechanisms have been proposed to explain how $A\beta(1-42)$ might block LTP, including endocytosis of synaptic NMDARs (Snyder et al., 2005; Goto et al., 2006; Kurup et al., 2010) and glutamate spillover resulting in overactivation of extrasynaptic NMDARs (O'Shea et al., 2008; Li et al., 2009), and in particular GluN2B-containing NMDARs (de Felice et al., 2007; Shankar et al., 2007; Ferreira et al., 2012; Li et al., 2011; Mota et al., 2014). Previous data from our laboratory indicate that both $A\beta(1-42)$ and monastrol result in reduced surface levels of NMDAR GluN1 and GluN2B subunits in cultured cells over several days, as measured by flow cytometry and confocal imaging (Ari et al., 2014). However, our data here are not consistent with any acute effect of $A\beta(1-42)$ or monastrol on synaptic NMDARs being responsible for LTP inhibition, as 20 minutes of preincubation with $A\beta(1-42)$ or monastrol was sufficient to inhibit LTP but had no significant impact on pharmacologically isolated *N*-methyl-D-aspartate fEPSPs. Indeed, $A\beta(1-42)$ showed no inhibition and monastrol only caused partial ($\sim 20\text{--}25\%$) inhibition of NMDAR responses even after 60 minutes. However, previous work by Raymond et al. (2003) did observe

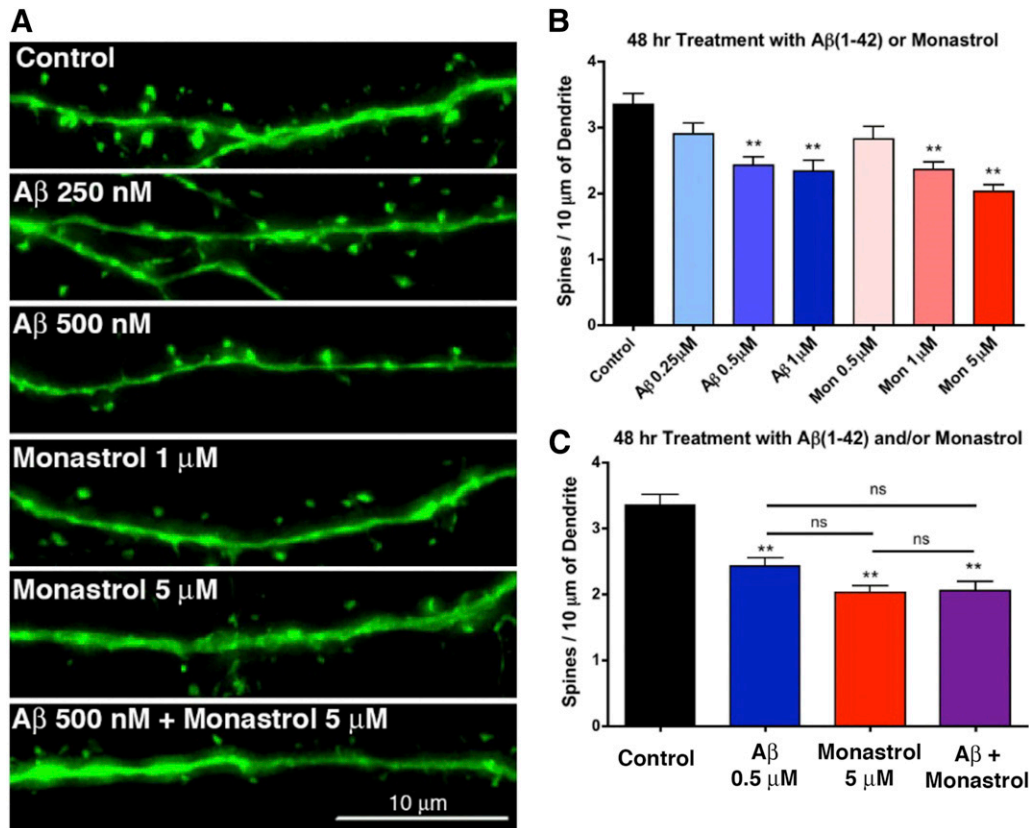


Fig. 5. Dendritic spine loss after incubation of cultured mouse hippocampal neurons with A β (1–42), monastrol, or both. (A) Neurons were transfected at 11 to 12 days in vitro to express green fluorescent protein (GFP) for visualization of dendritic spines. Neurons were left untreated (control) or treated with the indicated concentrations of A β (1–42) or monastrol or both for 48 hours before fixation at 14 to 15 days in vitro. GFP fluorescence in dendrites was imaged by acquiring three-dimensional image *z*-stacks (500 nm spacing) that were deconvolved to the nearest-neighbor and then collapsed into two-dimensional maximum intensity projection images. (B) Dendritic spine density was then quantified from projection images as the number of dendritic spines/10 μ m length of dendrite. (C) Maximal spine loss was obtained at 0.5 μ M A β (1–42) and 5 μ M monastrol; when applied together, no additional spine loss was observed. In Fig. 5C, data graphed for control, 0.5 μ M A β (1–42), and 5 μ M monastrol alone are the same as in Fig. 5B. We analyzed $n = 17$ –34 lengths of dendrite per condition. Data are the mean \pm S.E.M. * $P < 0.05$, ** $P < 0.01$ versus control by analysis of variance with Dunnett's post hoc.

~25% inhibition of NMDAR fEPSPs with only a 20-minute application of 200 nM A β (1–40). Yet, even in that study partial inhibition of NMDARs could not account for the complete inhibition of LTP by A β , because direct antagonism of NMDARs producing comparable inhibition of NMDAR fEPSPs did not alter LTP (Raymond et al., 2003). In addition, both our findings here and those of Raymond et al. (2003) are consistent with another previous study that observed acute A β (1–42) inhibition of LTP without any significant inhibition of synaptic NMDAR currents in whole-cell recordings of CA1 neurons (Nomura et al., 2005). Thus, longer applications of A β (1–42) than 60 minutes are likely necessary to observe substantial reductions in synaptic NMDAR responses as documented in other previous studies (Hsieh et al., 2006; Shankar et al., 2007).

Another process important for both synapse maintenance and LTP expression is the trafficking of AMPARs and other synaptic proteins to and from the synapse (Huganir and Nicoll, 2013). Our previous work indicated that A β (1–42) increased chromosome missegregation and aneuploidy (Granic et al., 2010) in cells due to A β (1–42) interfering with correct mitotic spindle formation through inhibiting the Eg5 microtubule motor (Borysov et al., 2011). Importantly, Eg5 is also involved in regulating the organization and stability of microtubules in axons and dendrites, and thus may also

indirectly impact microtubule-dependent receptor trafficking (Nadar et al., 2008; Ari et al., 2014; Kahn et al., 2015).

We previously found that a high concentration of monastrol (100 μ M) inhibited LTP (Ari et al., 2014). We confirmed that result here and further found that much lower concentrations of monastrol from 100 nM to 1 μ M also inhibit LTP to levels that are comparable to those observed with as little as 20–100 nM A β (1–42). Importantly, 50 nM A β (1–42) was previously shown to directly inhibit the activity of Eg5 and two other related motor proteins, KIF4A and MCAK (Borysov et al., 2011). However, interestingly we observed LTP inhibition with as little as 100 nM monastrol, which is below the low μ M IC₅₀ values previously measured for direct inhibition of Eg5 motor domain activity in vitro (Cochran et al., 2005). However, LTP is a very complex, nonlinear phenomenon, and we do not yet know how Eg5 function is integrated into its underlying mechanisms.

Thus, it is difficult to predict the sensitivity of LTP to inhibition by monastrol, or even A β , based solely on extrapolation from in vitro biochemical studies with isolated protein domains. For instance, it is possible that monastrol inhibition of LTP could involve not only direct inhibition of Eg5 motor activity but also downstream changes in the functions of other microtubule regulatory proteins such as tau, which is known to mediate some, but not all, of the impact of A β on neuronal

functions (reviewed in Mucke and Selkoe, 2012), including membrane trafficking (Umeda et al., 2015; Vossel et al., 2015). Accordingly, a recent study found that tau can also inhibit Eg5 activity; thus, A β may inhibit Eg5 not only directly but also indirectly via regulation of tau (Bougé and Parmentier, 2016). Overall, it is likely that there is very complex interplay between A β , tau, and Eg5 in the regulation of neuronal function, which definitely warrants future investigation.

Regulation of AMPAR trafficking, in particular, has received considerable attention in the mechanisms of A β -induced neuronal dysfunction because A β (1–42) can promote AMPAR endocytosis to favor synaptic depression (Hsieh et al., 2006). Indeed, application of A β (1–42) has been observed to result in reductions of surface AMPARs and AMPAR synaptic currents; reduced levels of surface AMPARs have also been observed in AD transgenic mice (Almeida et al., 2005; Roselli et al., 2005; Snyder et al., 2005; Hsieh et al., 2006; Zhao et al., 2010). Yet in our LTP experiments we found no decrease in AMPAR basal transmission during ~20 minutes of A β or monastrol preincubation before LTP induction. In addition, even when applied for 60 minutes, neither A β nor monastrol caused a decrease in AMPAR fEPSPs. Thus, direct promotion of AMPAR synaptic depression did not contribute to the LTP inhibition we observed for A β or monastrol. However, the substantial dendritic spine loss we observed with chronic A β or monastrol treatment is likely to be associated with glutamate receptor endocytosis and suppression of both AMPAR and NMDAR-mediated synaptic transmission, as seen in previous studies examining spine loss and synaptic dysfunction in neuronal cultures chronically exposed to A β (1–42) (Hsieh et al., 2006; Shankar et al., 2007).

Because we found that low-moderate concentrations of either A β (1–42) or monastrol were sufficient to completely inhibit LTP on their own, we were unable to clearly test whether LTP inhibition by cotreatment with A β oligomers and monastrol shows reciprocal occlusion (i.e., acting exclusively through the same mechanisms). However, lower doses of A β (1–42) (1 nM) and monastrol (50 nM), which at best partially inhibited LTP on their own, resulted in much more pronounced LTP inhibition when administered together. In other words, monastrol-mediated inhibition of Eg5 appeared to sensitize CA1 synapses to the inhibitory effect of A β (1–42) on LTP. Importantly, while monastrol and A β both inhibit Eg5 ATPase-dependent motor activity through mechanisms that are competitive with respect to regulation by microtubules, monastrol slows release of ADP and A β competes with ATP binding (Cochran et al., 2005; Borysov et al., 2011). Thus, additive inhibition could be expected for low concentrations of monastrol and A β even if both are converging on Eg5. In addition, A β (but not monastrol) inhibits the related motor proteins KIF4A and MCAK, which are both also expressed in neurons and could share functions with Eg5 that are required for LTP (Borysov et al., 2011).

Regardless of these remaining uncertainties for acute LTP inhibition, our comparison of the impacts of chronic cotreatment with A β (1–42) and monastrol on dendritic spine loss in cultured hippocampal neurons provided even clearer evidence that A β and monastrol share common downstream molecular mechanisms. In particular, we observed no additional spine loss after cotreatment with higher doses of monastrol (5 μ M) and A β (1–42) (0.5 μ M) that each alone triggered a maximum of 35% to 40% spine loss. Thus, overall, we found that monastrol can mimic, and in some cases occlude, the impacts

of A β (1–42) on excitatory synapses, suggesting that A β (1–42) may induce both acute and chronic synaptic dysfunction in part through inhibiting Eg5.

Dendritic spine loss is observed in both the cortical and hippocampal regions in humans with AD. In addition, spine loss and hippocampal LTP impairments are seen even at the very earliest stages of AD in mouse models when learning and memory alterations are also first evident, but before amyloid plaque and neurofibrillary tangles are observed (reviewed in Mucke and Selkoe, 2012). Our findings that the microtubule-dependent motor Eg5 is inhibited by A β oligomers and that direct Eg5 inhibition by monastrol can closely phenocopy both acute A β LTP inhibition and chronic A β -induced spine loss suggest that A β may disrupt microtubule-dependent neuronal functions, not only through its known regulation of tau but also through its inhibition of Eg5. In particular, inhibiting Eg5 may have a variety of impacts on the dendritic cytoskeleton that directly or indirectly alters spine structure, synaptic function, and LTP during even the earliest stages of AD. These results also suggest that blocking A β inhibition of Eg5/kinesin-5 could provide a novel approach for developing AD therapies.

Authorship Contributions

Participated in research design: Potter, Freund, Dell'Acqua.

Conducted experiments: Gibson, Freund.

Performed data analysis: Freund, Dell'Acqua, Gibson.

Wrote or contributed to the writing of the manuscript: Freund, Potter, Dell'Acqua.

References

- Almeida CG, Tampellini D, Takahashi RH, Greengard P, Lin MT, Snyder EM, and Gouras GK (2005) β -amyloid accumulation in APP mutant neurons reduces PSD-95 and GluR1 in synapses. *Neurobiol Dis* 20:187–198.
- Ari C, Borysov SI, Wu J, Padmanabhan J, and Potter H (2014) Alzheimer amyloid beta inhibition of Eg5/kinesin 5 reduces neurotrophin and/or transmitter receptor function. *Neurobiol Aging* 35:1839–1849.
- Baas PW (1998) The role of motor proteins in establishing the microtubule arrays of axons and dendrites. *J Chem Neuroanat* 14:175–180.
- Borysov SI, Granic A, Padmanabhan J, Walczak CE, and Potter H (2011) Alzheimer A β disrupts the mitotic spindle and directly inhibits mitotic microtubule motors. *Cell Cycle* 10:1397–1410.
- Bougé AL and Parmentier ML (2016) Tau excess impairs mitosis and kinesin-5 function, leading to aneuploidy and cell death. *Dis Model Mech* 9:307–319.
- Chen Q-S, Kagan BL, Hirakura Y, and Xie C-W (2000) Impairment of hippocampal long-term potentiation by Alzheimer amyloid β -peptides. *J Neurosci Res* 60:65–72.
- Cochran JC, Gatial JE, 3rd, Kapoor TM, and Gilbert SP (2005) Monastrol inhibition of the mitotic kinesin Eg5. *J Biol Chem* 280:12658–12667.
- Dickson DW, Crystal HA, Bevana C, Honer W, Vincent I, and Davies P (1995) Correlations of synaptic and pathological markers with cognition of the elderly. *Neurobiol Aging* 16:285–298, discussion 298–304.
- De Felice FG, Velasco PT, Lambert MP, Viola K, Fernandez SJ, Ferreira ST, and Klein WL (2007) Abeta oligomers induce neuronal oxidative stress through an N-methyl-D-aspartate receptor-dependent mechanism that is blocked by the Alzheimer drug memantine. *J Biol Chem* 282:11590–11601.
- Ferhat L, Cook C, Chauviere M, Harper M, Kress M, Lyons GE, and Baas PW (1998) Expression of the mitotic motor protein Eg5 in postmitotic neurons: implications for neuronal development. *J Neurosci* 18:7822–7835.
- Ferreira IL, Bajouco LM, Mota SI, Auberson YP, Oliveira CR, and Rego AC (2012) Amyloid beta peptide 1–42 disturbs intracellular calcium homeostasis through activation of GluN2B-containing N-methyl-D-aspartate receptors in cortical cultures. *Cell Calcium* 51:95–106.
- Goto Y, Nidome T, Akaike A, Kihara T, and Sugimoto H (2006) Amyloid beta-peptide preconditioning reduces glutamate-induced neurotoxicity by promoting endocytosis of NMDA receptor. *Biochem Biophys Res Commun* 351:259–265.
- Granic A, Padmanabhan J, Norden M, and Potter H (2010) Alzheimer A β peptide induces chromosome mis-segregation and aneuploidy, including trisomy 21: requirement for tau and APP. *Mol Biol Cell* 21:511–520.
- Hsieh H, Boehm J, Sato C, Iwatsubo T, Tomita T, Sisodia S, and Malinow R (2006) AMPAR removal underlies A β -induced synaptic depression and dendritic spine loss. *Neuron* 52:831–843.
- Huganir RL and Nicoll RA (2013) AMPARs and synaptic plasticity: the last 25 years. *Neuron* 80:704–717.
- Kahn OI, Sharma V, González-Billault C, and Baas PW (2015) Effects of kinesin-5 inhibition on dendritic architecture and microtubule organization. *Mol Biol Cell* 26:66–77.

- Keith DJ, Sanderson JL, Gibson ES, Woolfrey KM, Robertson HR, Olszewski K, Kang R, El-Husseini A, and Dell'acqua ML (2012) Palmitoylation of A-kinase anchoring protein 79/150 regulates dendritic endosomal targeting and synaptic plasticity mechanisms. *J Neurosci* **32**:7119–7136.
- Kim T, Vidal GS, Djuricic M, William CM, Birnbaum ME, Garcia KC, Hyman BT, and Shatz CJ (2013) Human LihR2 is a β -amyloid receptor and its murine homolog PirB regulates synaptic plasticity in an Alzheimer's model. *Science* **341**:1399–1404.
- Kimura R, MacTavish D, Yang J, Westaway D, and Jhamandas JH (2012) Beta amyloid-induced depression of hippocampal long-term potentiation is mediated through the amylin receptor. *J Neurosci* **32**:17401–17406.
- Kurup P, Zhang Y, Xu J, Venkataramani DV, Haroutunian V, Greengard P, Nairn AC, and Lombroso PJ (2010) Abeta-mediated NMDA receptor endocytosis in Alzheimer's disease involves ubiquitination of the tyrosine phosphatase STEP61. *J Neurosci* **30**:5948–5957.
- Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, Morgan TE, Rozovsky I, Trommer B, and Viola KL et al. (1998) Diffusible, nonfibrillar ligands derived from A β 1–42 are potent central nervous system neurotoxins. *Proc Natl Acad Sci USA* **95**:6448–6453.
- Laurén J, Gimbel DA, Nygaard HB, Gilbert JW, and Strittmatter SM (2009) Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. *Nature* **457**:1128–1132.
- Li S, Hong S, Shepardson NE, Walsh DM, Shankar GM, and Selkoe D (2009) Soluble oligomers of amyloid β protein facilitate hippocampal long-term depression by disrupting neuronal glutamate uptake. *Neuron* **62**:788–801.
- Li S, Jin M, Koeglsperger T, Shepardson NE, Shankar GM, and Selkoe DJ (2011) Soluble A β oligomers inhibit long-term potentiation through a mechanism involving excessive activation of extrasynaptic NR2B-containing NMDA receptors. *J Neurosci* **31**:6627–6638.
- Liu Q, Huang Y, Shen J, Steffensen S, and Wu J (2012) Functional α 7 β 2 nicotinic acetylcholine receptors expressed in hippocampal interneurons exhibit high sensitivity to pathological level of amyloid β peptides. *BMC Neurosci* **13**:155.
- McLean CA, Cherny RA, Fraser FW, Fuller SJ, Smith MJ, Beyreuther K, Bush AI, and Masters CL (1999) Soluble pool of A β amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. *Ann Neurol* **46**:860–866.
- Maliga Z, Kapoor TM, and Mitchison TJ (2002) Evidence that monastrol is an allosteric inhibitor of the mitotic kinesin Eg5. *Chem Biol* **9**:989–996.
- Mota SI, Ferreira IL, and Rego AC (2014) Dysfunctional synapse in Alzheimer's disease—a focus on NMDA receptors. *Neuropharmacology* **76** (Pt A):16–26.
- Mucke L and Selkoe DJ (2012) Neurotoxicity of amyloid β -protein: synaptic and network dysfunction. *Cold Spring Harb Perspect Med* **2**:a006338.
- Näslund J, Haroutunian V, Mohs R, Davis KL, Davies P, Greengard P, and Buxbaum JD (2000) Correlation between elevated levels of amyloid β -peptide in the brain and cognitive decline. *JAMA* **283**:1571–1577.
- Nadar VC, Ketschek A, Myers KA, Gallo G, and Baas PW (2008) Kinesin-5 is essential for growth-cone turning. *Curr Biol* **18**:1972–1977.
- O'Shea SD, Smith IM, McCabe OM, Cronin MM, Walsh DM, and O'Connor WT (2008) Intracerebroventricular administration of amyloid β -protein oligomers selectively increases dorsal hippocampal dialysate glutamate levels in the awake rat. *Sensors (Basel Switzerland)* **8**:7428–7437.
- Nomura I, Kato N, Kita T, and Takechi H (2005) Mechanism of impairment of long-term potentiation by amyloid β is independent of NMDA receptors or voltage-dependent calcium channels in hippocampal CA1 pyramidal neurons. *Neurosci Lett* **391**:1–6.
- Raymond CR, Ireland DR, and Abraham WC (2003) NMDA receptor regulation by amyloid- β does not account for its inhibition of LTP in rat hippocampus. *Brain Res* **968**:263–272.
- Robertson HR, Gibson ES, Benke TA, and Dell'Acqua ML (2009) Regulation of postsynaptic structure and function by an A-kinase anchoring protein-membrane-associated guanylate kinase scaffolding complex. *J Neurosci* **29**:7929–7943.
- Roselli F, Tirard M, Lu J, Hutzler P, Lamberti P, Livrea P, Morabito M, and Almeida OFX (2005) Soluble β -amyloid1-40 induces NMDA-dependent degradation of postsynaptic density-95 at glutamatergic synapses. *J Neurosci* **25**:11061–11070.
- Selkoe DJ (2008) Soluble oligomers of the amyloid β -protein impair synaptic plasticity and behavior. *Behav Brain Res* **192**:106–113.
- Shankar GM, Bloodgood BL, Townsend M, Walsh DM, Selkoe DJ, and Sabatini BL (2007) Natural oligomers of the Alzheimer amyloid-beta protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. *J Neurosci* **27**:2866–2875.
- Shankar GM, Li S, Mehta TH, Garcia-Munoz A, Shepardson NE, Smith I, Brett FM, Farrell MA, Rowan MJ, and Lemere CA et al. (2008) Amyloid- β protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. *Nat Med* **14**:837–842.
- Snyder EM, Nong Y, Almeida CG, Paul S, Moran T, Choi EY, Nairn AC, Salter MW, Lombroso PJ, and Gouras GK et al. (2005) Regulation of NMDA receptor trafficking by amyloid-beta. *Nat Neurosci* **8**:1051–1058.
- Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R, Hansen LA, and Katzman R (1991) Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. *Ann Neurol* **30**:572–580.
- Um JW, Kaufman AC, Kostylev M, Heiss JK, Stagi M, Takahashi H, Kerrisk ME, Vortmeyer A, Wisniewski T, and Koleske AJ et al. (2013) Metabotropic glutamate receptor 5 is a coreceptor for Alzheimer β oligomer bound to cellular prion protein. *Neuron* **79**:887–902.
- Umeda T, Ramser EM, Yamashita M, Nakajima K, Mori H, Silverman MA, and Tomiyama T (2015) Intracellular amyloid β oligomers impair organelle transport and induce dendritic spine loss in primary neurons. *Acta Neuropathol Commun* **3**:51.
- Vossel KA, Xu JC, Fomenko V, Miyamoto T, Suberbielle E, Knox JA, Ho K, Kim DH, Yu G-Q, and Mucke L (2015) Tau reduction prevents A β -induced axonal transport deficits by blocking activation of GSK3 β . *J Cell Biol* **209**:419–433.
- Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, Rowan MJ, and Selkoe DJ (2002) Naturally secreted oligomers of amyloid β protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* **416**:535–539.
- Wang HY, Lee DH, D'Andrea MR, Peterson PA, Shank RP, and Reitz AB (2000a) β -Amyloid(1-42) binds to α 7 nicotinic acetylcholine receptor with high affinity. Implications for Alzheimer's disease pathology. *J Biol Chem* **275**:5626–5632.
- Wang HY, Lee DH, Davis CB, and Shank RP (2000b) Amyloid peptide A β (1-42) binds selectively and with picomolar affinity to α 7 nicotinic acetylcholine receptors. *J Neurochem* **75**:1155–1161.
- Wimo A and Prince M (2011) World Alzheimer Report 2010: The global economic impact of dementia. Alzheimer's Disease International [online], <http://www.alz.co.uk/research/files/WorldAlzheimerReport2010.pdf>.
- Zhao W-Q, Santini F, Breese R, Ross D, Zhang XD, Stone DJ, Ferrer M, Townsend M, Wolfe AL, and Seager MA et al. (2010) Inhibition of calcineurin-mediated endocytosis and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors prevents amyloid β oligomer-induced synaptic disruption. *J Biol Chem* **285**:7619–7632.

Address correspondence to: Dr. Ronald K. Freund, Department of Pharmacology, University of Colorado School of Medicine, Anschutz Medical Campus, 12800 E. 19th Avenue, Mail Stop 8303, Aurora, CO 80045. E-mail: ron.freund@ucdenver.edu or Dr. Mark L. Dell'Acqua, Department of Pharmacology, 12800 E. 19th Avenue, Mail Stop 8303, Aurora, CO 80045. E-mail: mark.dellacqua@ucdenver.edu