

Intracellular Accumulation of Amyloid- β ($A\beta$) Protein Plays a Major Role in $A\beta$ -Induced Alterations of Glutamatergic Synaptic Transmission and Plasticity

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Intracellular accumulation of amyloid- β ($A\beta$) protein has been proposed as an early event in AD pathogenesis. In patients with mild cognitive impairment, intraneuronal $A\beta$ immunoreactivity was found especially in brain regions critically involved in the cognitive deficits of AD. Although a large body of evidence demonstrates that $A\beta$ 42 accumulates intraneuronally ($_{in}A\beta$), the action and the role of $A\beta$ 42 buildup on synaptic function have been poorly investigated. Here, we demonstrate that basal synaptic transmission and LTP were markedly depressed following $A\beta$ 42 injection into the neuron through the patch pipette. Control experiments performed with the reverse peptide ($A\beta$ 42-1) allowed us to exclude that the effects of $_{in}A\beta$ depended on changes in oncotic pressure. To further investigate $_{in}A\beta$ synaptotoxicity we used an $A\beta$ variant harboring oxidized methionine in position 35 that does not cross the neuronal plasma membrane and is not uploaded from the extracellular space. This $A\beta$ 42 variant had no effects on synaptic transmission and plasticity when applied extracellularly, but induced synaptic depression and LTP inhibition after patch-pipette dialysis. Finally, the injection of an antibody raised against human $A\beta$ 42 (6E10) in CA1 pyramidal neurons of mouse hippocampal brain slices and autaptic microcultures did not, per se, significantly affect LTP and basal synaptic transmission, but it protected against the toxic effects of extracellular $A\beta$ 42. Collectively, these findings suggest that $A\beta$ 42-induced impairment of glutamatergic synaptic function depends on its internalization and intracellular accumulation thus paving the way to a systemic proteomic analysis of intracellular targets/partners of $A\beta$ 42.

Key words: 6E10; amyloid- β protein; autaptic hippocampal neurons; intraneuronal accumulation; synaptic transmission; whole-cell LTP

Introduction

Amyloid- β ($A\beta$) oligomers have been proposed to be key mediators of cognitive decline in AD (Selkoe, 2002). $A\beta$ induces synaptotoxicity regardless of the genetic predisposition to this pathology (Li et al., 2013). Indeed, brain slices of *wt* mice exposed to nanomolar concentrations of human $A\beta$ showed impaired hippocampal LTP (Malenka and Malinow, 2011; Li et al., 2013), that is the cellular correlate of memory (Bliss and Collingridge, 1993; Nabavi et al., 2014). Many studies have proposed that $A\beta$ oligomers exert their synaptotoxic effects by binding to membrane receptors thereby affecting molecular pathways involved in neuronal functions responsible for the transmission and storage of information in the brain (Parihar and Brewer, 2010; Benilova et al., 2012; Benilova and De Strooper, 2013). However, to date

pharmacological approaches targeting these receptors have not yet led to effective treatments for preventing and/or delaying the disease progression (Small et al., 2007; Bonda et al., 2010). While several studies have investigated the effects of $A\beta$ on neuronal membrane receptors and the intracellular pathways activated downstream, the hypothesis that $A\beta$ 42 internalization from the extracellular space and its intraneuronal accumulation are critical to $A\beta$ 42-dependent synaptotoxicity has not been fully tested yet. Notably, in the early phases of AD (i.e., mild cognitive impairment and prodromal AD), intraneuronal accumulation of $A\beta$ was found especially in brain regions critically involved in the cognitive deficits (LaFerla et al., 2007). Moreover, the largest known genetic risk factor for late-onset sporadic AD, i.e., the ApoE4 isoform of ApoE gene, significantly increased $A\beta$ accumulation in neurons compared with the protective ApoE2 variant (Kuszczyk et al., 2013). There is also evidence that intraneuronal $A\beta$ accumulation contributes to tau hyperphosphorylation (Takahashi et al., 2010), reduces synaptic protein expression (Almeida et al., 2005), and induces mitochondrial dysfunction (Lustbader et al., 2004; Zepa et al., 2011). We recently demonstrated that 20 min of extracellular application of 200 nM $A\beta$ 42 decreased mEPSC frequency and vesicular release probability in autaptic hippocampal neurons without significantly affecting the postsynaptic compartment (Ripoli et al.,

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2013). We speculated that presynaptic alterations represent the earliest dysfunction followed by frank synapse loss. Here we report that intracellular accumulation of A β dramatically affects glutamatergic synaptic function at both presynaptic and postsynaptic levels. Our findings suggest that synaptotoxicity of A β may occur independently of its interaction with plasma membrane receptors, and that A β 42 internalization from the extracellular space and its intracellular accumulation play a pivotal role in synaptic dysfunction.

Materials and Methods

All animal procedures were approved by the Ethics Committee of the Catholic University and University of Catania, complied with Italian Ministry of Health guidelines and with national laws (Legislative decree 116/1992) and European Union guidelines on animal research (No. 86/609/EEC).

Preparation of amyloid solutions. Freeze-dried purified A β 40, A β 42, A β 42-1, and A β 42 variant harboring oxidized methionine at position 35 (A β 42^{MO}) and human amylin were purchased from AnaSpec. Protein solutions were prepared as previously described (Piacentini et al., 2008a; De Chiara et al., 2010; Maiti et al., 2011; Attar et al., 2012; Ripoli et al., 2013) according to standard procedures. Briefly, peptides were diluted to 1 mM in 1,1,1,3,3,3-hexafluoro-2-propanol to disassemble preformed aggregates and stored as dry films at -20°C before use. The films were dissolved at 1 mM in DMSO, sonicated for 10 min, diluted to 100 μM in cold PBS, and incubated for 12–18 h at 4°C to promote protein oligomerization. The final working concentrations (1–1000 nM) were obtained by diluting the 100 μM amyloid proteins in extracellular or intracellular solutions (for salts composition, see below). The same amount of DMSO/PBS contained in A β 42 solutions was used as vehicle. In some experiments, A β diluted in internal solution was subjected to 0.22 μm filtration (Minisart; Sartorius Stedim Biotech).

Western immunoblotting for A β 42. Protein solutions were analyzed by Western blotting as previously described with minor modification (De Stefano et al., 2005; Attar et al., 2012; Ripoli et al., 2013). Briefly, in SDS-PAGE analysis the A β samples (final concentration of 200 nM) were mixed with NuPAGE LDS sample buffer 4 \times and separated on 10–20% gradient Novex Tricine precast gels (Invitrogen) according to the manufacturer's protocol. After electrophoresis the proteins were transferred to 0.2 μm nitrocellulose membranes (GE Healthcare). Membranes were blocked for 1 h, at room temperature (RT; $22-24^{\circ}\text{C}$), in a suspension of 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 before incubation overnight at 4°C with mouse monoclonal antibody 6E10 (1:1000; Covance). Membranes were washed three times with Tris-buffered saline containing 0.1% Tween 20 and then incubated with HRP-conjugated anti-mouse secondary antibody (1:2000; Cell Signaling Technology) at RT for 1 h. Protein expression was evaluated by using the Super Signal West Femto chemiluminescence kit (Pierce). Immunoblots were documented by using UVItec Cambridge Alliance. Experiments were performed in triplicate.

Primary hippocampal neuron cultures. Hippocampal neurons from P0 to P2 C57BL/6 mice, eGFP-expressing mice (Okabe et al., 1997) or B6.129S7-App^{tm1Dbo/J} mice (APP knock-out mice purchased from The Jackson Laboratory) brains were prepared according to standard procedure as previously described (Piacentini et al., 2008b; Ripoli et al., 2013) with some modifications. Briefly, hippocampi were incubated for 10 min at 37°C in PBS containing trypsin-EDTA (0.025%/0.01% w/v; Biochrom AG), and the tissue was mechanically dissociated at RT with a fire-polished Pasteur pipette. The cell suspension was harvested and centrifuged at $235 \times g$ for 8 min. The pellet was suspended in 88.8% MEM (Biochrom), 5% fetal bovine serum, 5% horse serum, 1% glutamine (2 mM), 1% penicillin-streptomycin-neomycin antibiotic mixture (Invitrogen), and glucose (25 mM). Cells were plated at a density of 1×10^5 cells on 20 mm coverslips precoated with poly-L-lysine (0.1 mg/ml; Sigma). Twenty-four hours later, the culture medium was replaced with a mixture of 96.5% Neurobasal medium (Invitrogen), 2% B-27 (Invitrogen), 0.5% glutamine (2 mM), and 1% penicillin-streptomycin-neomycin antibiotic mixture. After 72 h, this medium was replaced with a glutamine-

free version of the same medium, and the cells were grown for 10 more days before experiments.

Electrophysiology in autaptic microcultures. Autaptic hippocampal neurons were prepared as previously described (Attar et al., 2012; Ripoli et al., 2013). To create microislands where glial cells could be grown, a mixture of poly-D-lysine and collagen (both from Sigma) was sprayed on agarose-coated glass coverslips. Cortical astrocytes from the brains of P0–P2 C57BL/6 mice (grown for 1 week in DMEM supplemented with 10% fetal bovine serum and antibiotics) were plated onto the coverslips (Podda et al., 2012). After 4–6 d, the medium was conditioned before neuron plating by replacing half the medium volume with neuronal medium (consisting of Neurobasal medium, 2% B-27, 0.5% glutamine, and 1% penicillin-streptomycin-neomycin antibiotic mixture). Hippocampal neurons from P0 to P2 C57BL/6 and B6.129S7-App^{tm1Dbo/J} mice were prepared as described above and suspended in neuronal medium. Later, neurons were plated onto glial microislands at low density (25,000/cm²) to obtain a ratio of one neuron per island. Every 4 d half the neuronal medium volume was replaced with fresh neuronal medium supplemented with 2 μM cytosine arabinoside. Autapses were studied from 9 to 21 DIV.

Basal synaptic transmission was studied using the patch-clamp technique in the whole-cell configuration as previously described (Attar et al., 2012; Ripoli et al., 2013). Recordings were obtained with an Axopatch 200B amplifier (Molecular Devices), and stimulation and data acquisition were performed with the Digidata 1200 series interface and pCLAMP 10 software (Molecular Devices). Patch electrodes, fabricated from borosilicate glass capillaries with the aid of a micropipette puller (P-97; Sutter Instruments) had resistances of 3–5 M Ω when filled with the internal solution that contained the following (in mM): 146 K-gluconate, 18 HEPES, 1 EGTA, 4.6 MgCl₂, 4 NaATP, 0.3 Na₂GTP, 15 creatine phosphate, and 5 U/ml phosphocreatine kinase. For recordings, cells were constantly perfused with an external Tyrode's solution containing the following (in mM): 140 NaCl, 2 KCl, 10 HEPES, 10 glucose, 4 MgCl₂, and 4 CaCl₂, pH 7.4, 312 mOsm. We monitored the access resistance and membrane capacity before and at the end of the experiments to ensure recording stability and the health of studied cells. Recordings were considered stable when the series and input resistances, resting membrane potential, and stimulus artifact duration did not change $>20\%$. Comparisons were performed between data collected after whole-cell configuration had been achieved (referred as T₀) and 20 min later (referred as T₂₀).

EPSCs were recorded in autaptic neurons voltage clamped at a membrane potential of -70 mV, with stimuli mimicking action potentials (2 ms at 0 mV) delivered every 10 s or 20 s. NMDA currents were evoked using Mg-free Tyrode's solution containing 10 μM of the AMPA receptor blocker NBQX (Tocris Bioscience). To obtain the AMPA:NMDA ratio, evoked responses were always recorded successively from the same cell. The amplitude and frequency of spontaneous mEPSCs were evaluated in 60 s recordings. The detection threshold was set to 3.5 times the baseline SD. The size of the readily releasable vesicle pool (RRP) of synaptic vesicles was estimated by measuring the charge induced by increasing the transmembrane osmotic pressure in the presynaptic terminal by a 4 s extracellular application of hypertonic (0.5 M) sucrose solution. The total RRP charge was then estimated as the integral of the fast, transient inward current component, after subtraction of the steady-state component. RRP refilling was investigated in paired-pulse experiments, in which 0.5 M sucrose solution was applied for 4 s at 4 s interpulse intervals. The RRP recovery rate was expressed as the peak amplitude of the second response normalized to that of the first response. To evaluate short-term plasticity, EPSCs were recorded from neurons stimulated at 20 Hz (Ting et al., 2006). The decay time constant (τ) was measured by fitting the normalized EPSC amplitude plot of each cell with monoexponential function (Origin 5.0 software; OriginLab). The paired-pulse ratio (PPR) consisted of the ratio of the amplitude of the second EPSC to that of the first recorded in 20 Hz trains. Steady-state values were evaluated by averaging EPSC amplitudes in response to 30–40th stimuli. All experiments were performed at RT.

Electrophysiology in hippocampal brain slices. All experiments were performed with 21-d-old male C57BL/6 mice. Animals were anesthetized

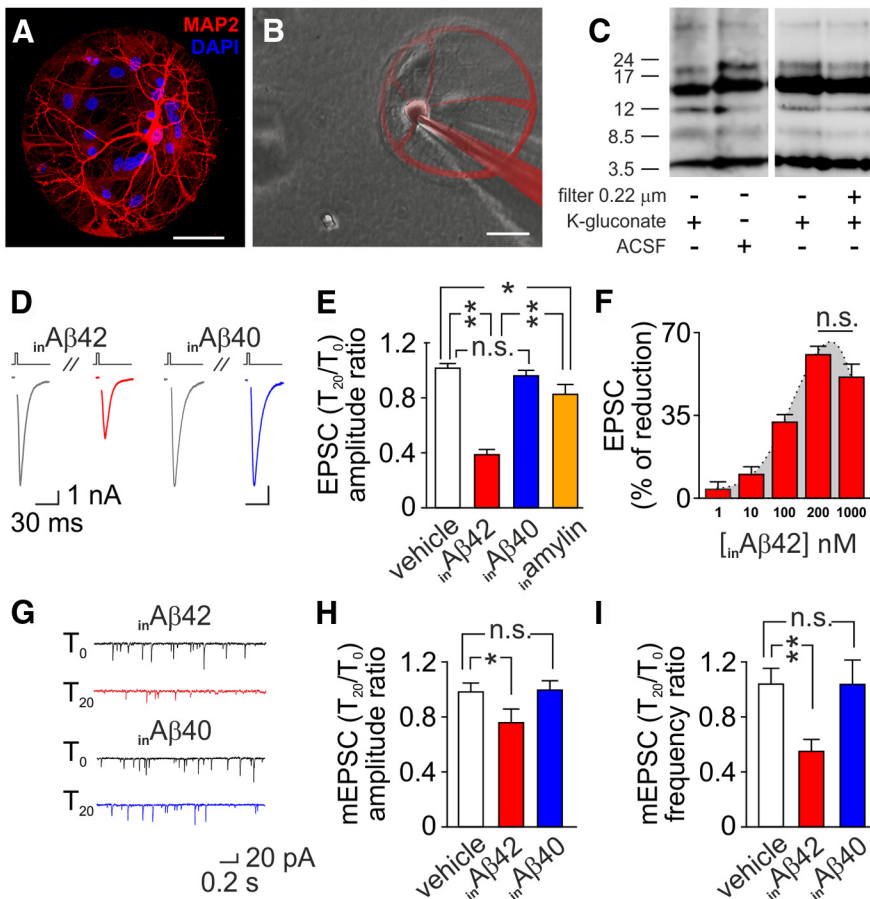


Figure 1. Accumulation of in A β plays a major role in A β -induced alterations of glutamatergic synaptic function. **A**, Representative image of a hippocampal autaptic culture. Red staining for MAP2 identifies the single neuron grown onto glial microisland. Blue staining (DAPI) identifies cell nuclei. **B**, Image depicting intracellular application of 200 nM A β 42. **C**, Representative Western blot of A β oligomer distribution in ACSF and unfiltered or 0.22 μ m filtered K-gluconate solution. None of the above described experimental conditions markedly affected A β 42 small oligomer distribution. **D**, Representative traces of EPSC currents at T_0 (gray lines) and after 20 min intracellular application of 200 nM A β 42 (red line) or 200 nM A β 40 (blue line). Stimulus artifacts for EPSC currents were removed for clarity. **E**, Bar graphs (mean \pm SEM) showing the T_{20}/T_0 ratio of EPSC amplitude in autaptic neurons exposed to vehicle (white bar), in A β 42 (red bar), in A β 40 (blue bar), or amylin (orange bar). **F**, Dose–response relationship of in A β 42 effects on EPSC amplitude. **G**, Representative traces of mEPSC currents at T_0 (gray lines) and T_{20} with 200 nM in A β 42 (red line) or 200 nM in A β 40 (blue line). Bar graphs (mean \pm SEM) showing the T_{20}/T_0 ratio of mEPSC amplitude (**H**) and frequency (**I**) in autaptic neurons exposed to vehicle (white bars), in A β 42 (red bars), or in A β 40 (blue bars). Scale bars: **A**, **B**, 50 μ m. * p < 0.05; ** p < 0.005; n.s.: p > 0.05.

with isoflurane and decapitated. The brains were rapidly removed and placed in ice-cold cutting solution containing the following (in mM): 124 NaCl, 3.2 KCl, 1 NaH $_2$ PO $_4$, 2 MgCl $_2$, 1 CaCl $_2$, 26 NaHCO $_3$, 10 glucose, 2 Na-pyruvate, and 0.6 ascorbic acid, pH 7.4, 95% O $_2$ /5% CO $_2$. Slices (300 μ m thick) were cut on a vibratome (VT1000S; Leica Microsystems) and immediately transferred to an incubation chamber filled with ACSF containing the following (in mM): 124 NaCl, 3.2 KCl, 1 NaH $_2$ PO $_4$, 1 MgCl $_2$, 2 CaCl $_2$, 26 NaHCO $_3$, and 10 glucose, pH 7.4, 95% O $_2$ /5% CO $_2$. The slices were allowed to recover at 32°C for 1 h before being equilibrated at RT. For electrophysiological recordings, slices were transferred to a submerged recording chamber constantly perfused with heated ACSF (32°C) and bubbled with 95% O $_2$ /5% CO $_2$ (Podda et al., 2008; Fusco et al., 2012).

Experiments examining LTP were performed from single CA1 pyramidal cells after stimulating the Schaffer collateral fibers by means of a bipolar tungsten electrode (Warner Instruments). All recordings were made with the GABA $_A$ receptor antagonist picrotoxin (50 μ M) added to the ACSF. Whole-cell recording pipettes (3–5 M Ω) were filled with a solution containing the following (in mM): 135 CsMeSO $_3$, 8 NaCl, 10 HEPES, 0.25 EGTA, 2 Mg $_2$ ATP, 0.3 Na $_3$ GTP, 0.1 spermine, 7 phosphocreatine, and 5 QX-314, pH 7.25–7.30, 294–298 mOsm. Data were col-

lected with a Multiclamp 700A amplifier (Molecular Devices). A Digidata 1440 series interface and pClamp 10 software (Molecular Devices) were used for data acquisition and stimulation protocols. Data were filtered at 1 kHz, digitized at 10 kHz, and analyzed on-line and off-line. Hippocampal subfields and electrode positions were identified with the aid of 4 \times and 40 \times water-immersion objectives on an upright microscope equipped with differential interference contrast optics under infrared illumination (BX51WI; Olympus) and video observation (C3077-71 CCD camera; Hamamatsu Photonics).

To study LTP in CA1 pyramidal cells, the amplitudes of EPSCs elicited by stimulation of Schaffer collateral fibers were measured. The stimulation intensity that elicited one-third of the maximal response was used for delivering test pulses every 20 s. During recordings, CA1 pyramidal cells were held at -60 mV to record AMPA receptor-mediated EPSCs. LTP was induced by two trains of HFS (100 Hz, 1 s) separated by 20 s, while cells were depolarized to 0 mV. This induction protocol was always applied within 5–7 min of achieving whole-cell configuration, to avoid “wash-out” of LTP. Responses to test pulse were recorded for 30 min to assess LTP. The amplitudes of EPSC at 30 min were averaged from values obtained during the last 5 min of post-HSF recordings (from minute 25 to minute 30). LTP magnitude was expressed as the percentage change in the mean EPSC peak amplitude normalized to baseline values = 100% (i.e., mean values for the 5 min of recording before HFS). Unless otherwise specified, all commercial products were used according to manufacturers’ instructions.

Study of A β internalization. To study internalization of A β 42, both *wt* and A β 42^{MO} were labeled with the IRIS 5-NHS active ester dye (IRIS 5; λ_{ex} : 633 nm, λ_{em} : 650–700 nm; Cyanine Technology) as previously described (Ripoli et al., 2013). IRIS 5 dye is suitable for conjugation of any biomolecules carrying free primary amines, such as proteins and peptides. Briefly, A β solutions (100 μ M in PBS) were mixed with 6 mM IRIS 5 in DMSO for 4 h in the dark under mild shaking conditions. After this

time, labeled A β s were purified with Vivacon 500 ultrafiltration spin columns (2 kDa cutoff; Sartorius Stedim Biotech) and then resuspended in PBS at a concentration of 100 μ M before final dilution in the culture medium.

Time-dependent internalization of IRIS 5-labeled A β 42 (either *wt* or A β 42^{MO}) was then studied by time-lapse confocal imaging in hippocampal neurons derived from eGFP-expressing mice or by immunocytochemistry in hippocampal neurons derived from C57BL/6 mice.

Immunocytochemistry. Hippocampal neurons cultured for 15 DIV and treated with IRIS 5-labeled A β 42 analogs were fixed with 4% paraformaldehyde (Sigma) in PBS for 15 min at RT. After being permeabilized (15 min incubation with 0.3% Triton X-100 in PBS; Sigma), cells were incubated for 20 min with 0.3% BSA in PBS to block nonspecific binding sites and then overnight at 4°C with mouse antimicrotubule-associated protein 2 (MAP2; 1:300; Sigma). For experiments aimed at evaluating the effects of A β 42 on synaptic proteins, neuronal cultures were fixed and permeabilized as previously described, and then incubated overnight with either mouse anti-PSD-95 (1:250; Abcam) or rabbit monoclonal anti-synaptophysin (1:250; Abcam) and mouse anti-MAP2. The next day, cells were incubated for 90 min at RT with Alexa Fluor 488 donkey

anti-mouse or donkey anti-rabbit and/or Alexa Fluor 546 donkey anti-mouse antibodies (1:1000; Invitrogen). Nuclei were counterstained with DAPI (0.5 μ g/ml for 10 min; Invitrogen), and finally cells were coverslipped with ProLong Gold antifade reagent (Invitrogen).

Images (512 \times 512 or 1024 \times 1024 pixels) were acquired at 63 \times magnification with a confocal laser scanning system (TCS-SP2; Leica) and an oil-immersion objective (NA 1.4). DAPI staining was imaged after two-photon excitation with an ultrafast, tunable mode-locked titanium:sapphire laser (Chameleon; Coherent).

Immunofluorescence for synaptophysin and PSD-95 was quantified as the sum of fluorescence intensities (8-bit depth) measured for every pixel in the recorded field. For synaptophysin, we also calculated the “protein density”, i.e., the total fluorescence intensity of synaptophysin labeling divided by the total area in the field that was occupied by neurons (identified by MAP2 immunoreactivity). The operator was blind to the study conditions.

Immunohistochemistry and biocytin labeling. To study dendritic spine density, patch-clamped CA1 neurons in hippocampal slices were dialyzed with the intracellular solutions containing 0.2% biocytin (Sigma) and either 200 nM $_{in}A\beta 42$ or vehicle. After 20 min of whole-cell dialysis, slices were fixed overnight at 4°C with 0.1 M PBS containing 4% paraformaldehyde. After fixation, slices were incubated for 60 min in blocking solution containing 10% normal goat serum and 0.3% Triton X-100 (Sigma) in PBS. Subsequently, biocytin was revealed by incubating slices with Alexa Fluor 488-conjugated avidin (1:500 in blocking solution; Life Technologies) for 90 min at RT. Slices were then washed three times in PBS, mounted with ProLong Gold antifade reagent (Life Technologies), and finally studied with a high-resolution confocal microscope (Leica TCS-SP2).

Spines were imaged with a 63 \times magnification objective (NA 1.40) plus additional magnification of 5 \times . Images were taken at 1024 \times 1024 pixel resolution (physical pixel size: 46 nm). Spine density was analyzed under blinded conditions in randomly chosen segments (length: 40–43 μ m) of secondary dendrites from apical branches and expressed as the number of spines per 100 μ m dendrites. A total length of at least 1.2 mm was analyzed for each experimental condition.

Apoptosis assays. Apoptosis was evaluated in hippocampal cultures with the APO-BrdU TUNEL assay Kit (Invitrogen) according to the manufacturer’s instructions, as described in Podda et al. (2014). Briefly, the cell cultures were exposed to 200 nM A $\beta 42$ or vehicle for 1 h. Cells exposed for 3 h to 100 μ M H₂O₂ were used as positive control of apoptosis. Apoptotic cells were identified immunocytochemically by means of anti-BrdU antibody labeled with Alexa Fluor 488 dye, whereas cell nuclei were identified by means of propidium iodide. Images (512 \times 512 pixels) were obtained at 40 \times magnification with a high-resolution confocal microscope (Leica TCS-SP2).

In some cultures, the intermediate stages of apoptosis were also investigated by annexin V staining (FITC conjugated; Life Technologies).

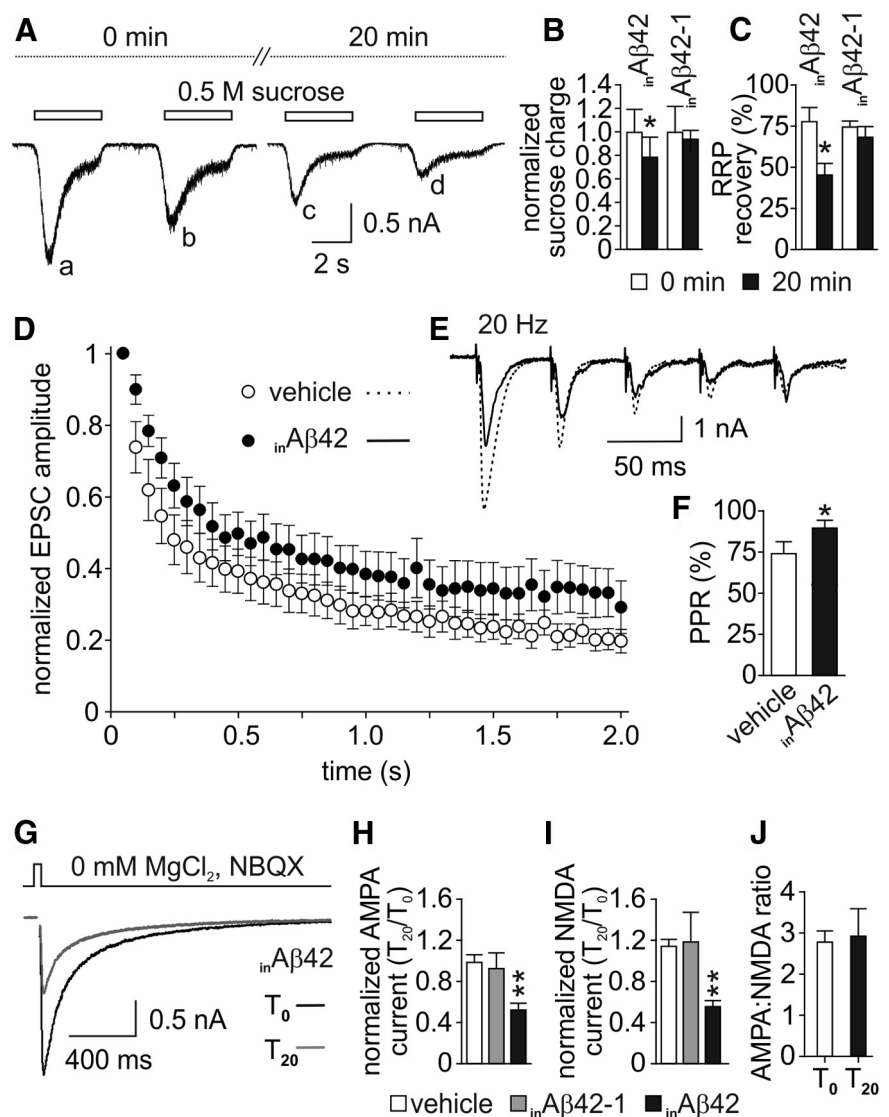


Figure 2. Intracellular application of A $\beta 42$ markedly affects glutamatergic synaptic transmission. **A**, Representative traces of paired-pulse currents induced by 4 s applications of 0.5 M sucrose (4 s interpulse intervals) at T_0 and T_{20} of $_{in}A\beta 42$. Following A $\beta 42$ injection (trace c collected at T_{20}) the sucrose charge was markedly lower than at T_0 (trace a). **B**, Bar graphs (mean \pm SEM) showing the normalized sucrose charge at T_0 (white bars) and T_{20} (black bars) in $_{in}A\beta 42$ - or $_{in}A\beta 42$ -1-injected neurons. **C**, Bar graphs (mean \pm SEM) showing the RRP recovery rate expressed as the peak amplitude of the second responses normalized to that of the first response at T_0 (b/a in **A**; white bars) and T_{20} (d/c in **A**; black bars) in $_{in}A\beta 42$ - or $_{in}A\beta 42$ -1-injected neurons. **D**, EPSC amplitudes normalized to the first response during 2 s trains at 20 Hz. **E**, Representative traces of the first five responses evoked by trains of stimuli (20 Hz) after 20 min of vehicle or $_{in}A\beta 42$ applications. **F**, Mean PPRs after 20 min of vehicle or $_{in}A\beta 42$ application. **G**, Representative traces of NMDA currents (stimulus artifacts removed for clarity) at T_0 (black line) and T_{20} (gray line) with $_{in}A\beta 42$. Bar graphs (mean \pm SEM) showing the T_{20}/T_0 ratio of AMPA (**H**) and NMDA (**I**) currents with vehicle (white bars), $_{in}A\beta 42$ -1 (gray bars), and $_{in}A\beta 42$ (black bars). **J**, Bar graphs (mean \pm SEM) showing the AMPA:NMDA ratio at T_0 and T_{20} with $_{in}A\beta 42$. * $p < 0.05$; ** $p < 0.01$.

Statistical analysis. All data are shown as mean \pm SEM. Statistical analyses (Student’s paired and unpaired t tests) were performed with SYSTAT 10.2 software (Statcom). The level of significance was set at 0.05.

Results

Intracellular application of A $\beta 42$ markedly affected glutamatergic synaptic transmission

In autaptic hippocampal neurons we studied the effects of intracellular application of A $\beta 42$ ($_{in}A\beta 42$), injected into neurons through the patch pipette (Fig. 1A,B). First, we checked whether the different composition of intrapipette and extracellular solutions we used for patch-clamp experiments affected A $\beta 42$ oligomerization. Western

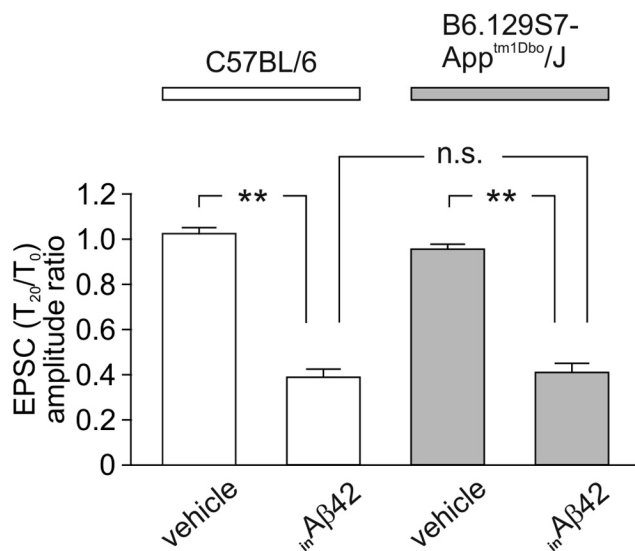


Figure 3. EPSC amplitude inhibition induced by $inA\beta 42$ is independent of its interaction with either APP or APP cleavage products. Bar graphs (mean \pm SEM) showing the T_{20}/T_0 ratio of EPSC amplitude in autaptic neurons derived from C57BL/6 (white bars) and B6.129S7-App^{tm1Db0}/J mice (APP knock-out mice; gray bars) exposed to either vehicle or 200 nM $inA\beta 42$. ** $p < 0.005$; n.s.: $p > 0.05$.

blot analysis showed similar A β oligomer distribution in the different solutions used (Fig. 1C). Synaptic strength was quantified by measuring the amplitude of action potential-evoked EPSCs along with mEPSC amplitude and frequency. EPSC amplitudes were markedly depressed after 20 min (T_{20}) application of 200 nM $inA\beta 42$ (T_{20} vs T_0 : $-62.3 \pm 3.1\%$; $n = 24$; $p < 0.005$; Fig. 1D,E), whereas after 20 min intracellular application of vehicle we found no significant changes in EPSC amplitudes ($p = 0.38$; $n = 22$; Fig. 1E).

To determine the specificity of $inA\beta 42$ effects we also loaded neurons with either 200 nM A $\beta 40$, another most common isoform of A β , or 200 nM A $\beta 42-1$ with the same molecular weight and amino acid composition of A $\beta 1-42$ but assembled in the reverse mode. Neither A $\beta 40$ nor A $\beta 42-1$ significantly affected EPSC amplitudes [T_{20} vs T_0 : $p = 0.49$ ($n = 10$) and 0.75 ($n = 11$), respectively; Fig. 1D,E]. Although A $\beta 42-1$ shares with A $\beta 1-42$ the same molecular weight and amino acid composition, neither this reverse peptide nor A $\beta 40$ exhibit the same tendency of A $\beta 42$ to oligomerize. We then performed further control experiments with amylin, an amyloid protein that differs from A $\beta 42$ in its primary sequence but shares with it the ability to oligomerize (Lorenzo et al., 2000). As expected on the basis of previous studies (Kimura et al., 2012), we found that 20 min intracellular application of 200 nM amylin decreased EPSC amplitude by $17.6 \pm 6.5\%$ ($p < 0.05$; $n = 11$; Fig. 1E). However, such decrease was significantly lower than that caused by 200 nM $inA\beta 42$ ($inA\beta 42$ vs $inamylin$; $p < 0.005$).

Dose–response relationship was studied by using $inA\beta 42$ concentrations ranging from 1 to 1000 nM (Fig. 1F), which are in the same range of intracellular A $\beta 42$ concentrations reported in neurons of AD models (Hu et al., 2009; Hashimoto et al., 2010). The lowest concentration causing a statistically significant decrease in EPSC amplitude was 10 nM ($-9.1 \pm 4.3\%$; $n = 21$; $p < 0.01$). Higher $inA\beta 42$ concentrations produced more marked effects reaching a plateau at 200 nM. Indeed, no significant differences were found between EPSC amplitude decreases observed at 200 and 1000 nM ($p = 0.11$; Fig. 1F).

As shown in Figure 1G–I, exposure to $inA\beta 42$ decreased both mEPSC amplitude (T_{20} vs T_0 : $-23.5 \pm 10.9\%$; $n = 16$; $p < 0.05$)

and frequency ($-44.8 \pm 8.4\%$; $n = 16$; $p < 0.005$). Neither vehicle ($n = 15$) nor 200 nM $inA\beta 40$ ($n = 10$) significantly modified these parameters (Fig. 1G–I). In a set of experiments aimed at investigating in greater detail the effects of $inA\beta 42$ on synaptic transmission, we challenged neurons with 0.5 M sucrose solution to measure the RRP at T_0 and 20 min after $inA\beta 42$ loading (Fig. 2A). Upon locally puffing 0.5 M sucrose for 4 s onto the recorded autaptic neuron, a transient current reflecting glutamate release from docked vesicles was observed (Fig. 2A). The response to this hypertonic stimulus was significantly smaller after 20 min $inA\beta 42$ application than at T_0 ($-23.7 \pm 8.4\%$; $p < 0.05$; $n = 8$; Fig. 2A, compare traces c and a, B). Paired stimuli of hypertonic sucrose solution, delivered at 4 s intervals, revealed a decreased refilling rate of the RRP after 20 min $inA\beta 42$ application (T_0 : $78.3 \pm 9.0\%$; T_{20} : $45.8 \pm 6.5\%$; $n = 8$; $p < 0.05$; Fig. 2A, C), likely reflecting a reduced vesicle number and/or vesicular release probability within terminals. Control experiments performed with 200 nM $inA\beta 42-1$ did not reveal significant changes in either sucrose charge or RRP recovery ($n = 8$; $p = 0.71$ and 0.16 , respectively; Fig. 2B, C). Moreover, we estimated the vesicular release probability and the short-term plasticity within these synapses by using a train of 40 stimuli at 20 Hz (Fig. 2D–F). The kinetics of EPSC amplitude depression was significantly slower after 20 min $inA\beta 42$ application relative to controls: the mean decay time constants (τ) of monoexponential functions fitting the normalized EPSC amplitude plots were 380 ± 72 ms in A $\beta 42$ -injected neurons and 220 ± 33 ms in controls ($n = 15$; $p < 0.05$). This finding suggests a reluctance of synapses in A $\beta 42$ -filled neurons to deplete the RRP vesicles. Release probability was investigated by analyzing the PPR between the firsts two stimuli of the above-mentioned 20 Hz paradigm that was significantly increased by $inA\beta 42$ ($p < 0.05$; Fig. 2D–F). These data suggest a significant decrease of release probability in A $\beta 42$ -injected neurons compared with controls.

As shown in Figure 2, G and I, NMDA currents were also significantly reduced 20 min after 200 nM $inA\beta 42$ ($-44.2 \pm 6.5\%$, $n = 10$; $p < 0.01$). In this set of experiments, we restricted our study to NMDA receptor- and AMPA receptor-mediated EPSC amplitudes without investigating other electrophysiological parameters to minimize the risk of current run-down occurring in long-lasting recordings. In the same neuron we first recorded the synaptically evoked NMDA currents followed by AMPA receptor-mediated EPSCs, both at T_0 and T_{20} . After 20 min $inA\beta 42$ the decrease in NMDA current amplitude was very similar to that observed in AMPA currents ($-44.2 \pm 6.5\%$ and $-47.6 \pm 6.4\%$, respectively; Fig. 2H, I). This finding was confirmed by studying the AMPA:NMDA ratio that was unaltered in hippocampal autapses after A $\beta 42$ loading via the patch pipette (Fig. 2J).

Literature reports suggested that A β toxicity may be mediated by its binding to APP (Lorenzo et al., 2000; Shaked et al., 2006). To check whether the diminished neurotransmission induced by $inA\beta 42$ depended of its interaction with either APP or APP cleavage products including A $\beta 42$ physiologically present in neurons, we performed patch-clamp recordings in autaptic hippocampal APP-null neurons loaded with 200 nM A $\beta 42$. The EPSC amplitude inhibition induced by $inA\beta 42$ in this experimental model ($-58.1 \pm 2.8\%$; $n = 17$; $p < 0.005$; Fig. 3) was not significantly different from that observed in hippocampal neurons from *wt* mice.

Finally, we checked whether $inA\beta 42$ synaptotoxicity was due to specific early synaptic effects rather than to cell death/damage or frank synapse loss. To this aim we studied: (1) dendritic spine density in hippocampal neurons of brain slices loaded with both

A β 42 and biocytin and (2) expression of synaptic proteins (synaptophysin and PSD-95) and apoptosis (annexin V immunoreactivity and TUNEL assay) in hippocampal neuronal cultures treated for 60 min with vehicle or A β 42. As shown in Figure 4, A–C, 20 min intracellular application of 200 nM A β 42 did not significantly affect dendritic spine density (79 ± 4 and 77 ± 5 spines per 100 μ m with vehicle and 200 nM $_{in}$ A β 42, respectively; $p = 0.65$). Moreover, study of annexin V immunoreactivity and TUNEL assay did not reveal signs of cell death/damage in neurons exposed for 60 min to extracellular A β 42 (data not shown). Noteworthy, as described in greater detail below, cell-culture exposure to 200 nM A β 42 for 60 min was associated with significant A β 42 internalization and intraneuronal accumulation. Finally, the same treatment did not significantly affect synaptophysin and PSD-95 immunoreactivity (data not shown).

Hippocampal LTP was markedly inhibited by $_{in}$ A β 42

To investigate the effects of $_{in}$ A β 42 on synaptic plasticity, in acute hippocampal brain slices we studied LTP at CA3–CA1 synapses by applying LTP protocols within 5–7 min after achieving whole-cell configuration (see Materials and Methods). Under control conditions, i.e., when hippocampal slices were perfused with vehicle alone or A β 42-1 was injected into CA1 pyramidal neurons, the EPSC amplitude recorded 30 min after HFS displayed increases of $119.7 \pm 17.8\%$ ($n = 8$) and $127.0 \pm 30.1\%$ ($n = 8$), respectively (Fig. 5A,B). LTP was markedly lower when neurons were loaded with 200 nM A β 42 through the patch pipette ($14.9 \pm 18.7\%$; $n = 8$; $p < 0.005$; Fig. 5A,B).

An A β 42 variant, unable to be uploaded intraneuronally, had no effects on synaptic transmission and plasticity when applied extracellularly but induced synaptic depression and LTP inhibition after patch-pipette dialysis

Numerous papers, including ours, demonstrated that extracellularly applied A β 42 ($_{ex}$ A β 42) markedly affects synaptic transmission and plasticity (Pettit et al., 2001; Wei et al., 2010; Jo et al., 2011; Attar et al., 2012; Li et al., 2013; Ripoli et al., 2013). We hypothesized that neuronal uploading of $_{ex}$ A β 42 from extracellular space and its intracellular accumulation are critical determinants of its synaptotoxicity. To test this hypothesis, we labeled A β 42 with the fluorescent dye IRIS 5-NHS and studied its localization by high-resolution confocal microscopy. We found that, when applied extracellularly, A β 42 crossed the neuronal membrane and was internalized both at somatic and dendritic levels during the 20 min application (Fig. 6A,B), which is the time frame of our electrophysiological recordings. We then looked for A β variants exhibiting biophysical properties comparable to those of *wt*A β but unable to cross plasma membrane and be uploaded intraneuronally. In previous studies we demonstrated that A β 42^{MO} exhibited soluble small-oligomer distribution similar to that of *wt*A β but very limited neurotoxicity (Piacentini et al., 2008a; Ripoli et al., 2013). This A β variant scarcely crossed the neuronal membrane: after 20 min application most of the labeled

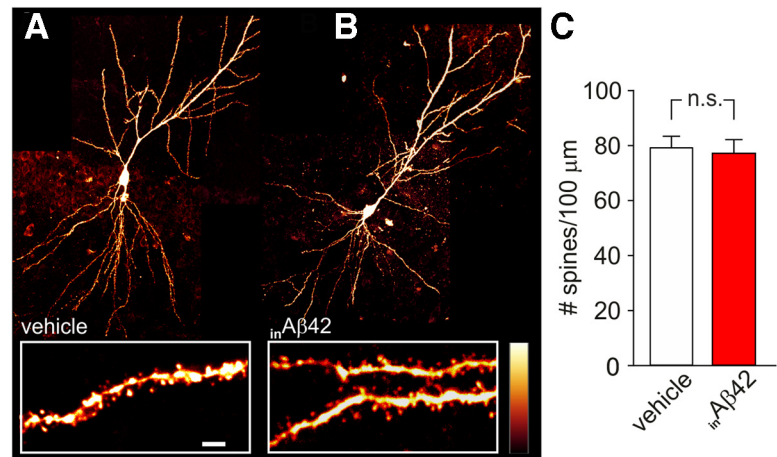


Figure 4. Twenty minute injection of A β 42 does not significantly affect dendritic spine density of CA1 hippocampal neurons. **A**, **B**, Representative examples of CA1 neurons filled with biocytin and revealed with avidin conjugated to Alexa Fluor 488. Neuron shown in **A** was injected with vehicle, whereas neuron shown in **B** was injected with 200 nM A β 42 for 20 min. Bottom boxes in **A** and **B** show high-magnification images of dendritic segments of cells in **A** and **B**, respectively. Scale bar, 3 μ m. Alexa Fluor 488 fluorescence intensity (8-bit depth) was represented according to the color scale on the right (bottom = 0, top = 256). **C**, Bar graphs showing the mean number of dendritic spines per 100 μ m. n.s.: $p > 0.05$.

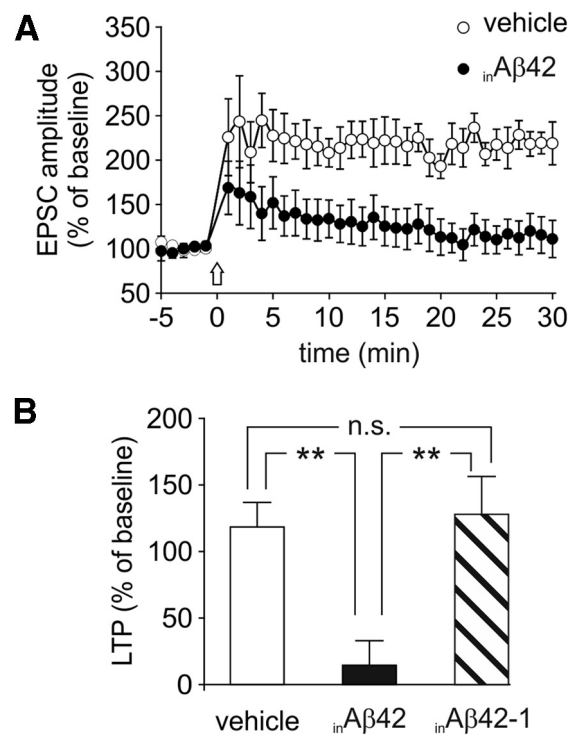


Figure 5. LTP is markedly inhibited by $_{in}$ A β 42. **A**, Time course of EPSC amplitudes before and after HFS (indicated by arrow) in hippocampal brain slices treated with vehicle (white circles) and $_{in}$ A β 42 (black circles). **B**, Bar graphs (mean \pm SEM) showing the EPSC amplitudes measured during the last 5 min of recording under the conditions described for **A** and during the last 5 min of $_{in}$ A β 42-1. ** $p < 0.005$; n.s.: $p > 0.05$.

A β 42^{MO} was confined outside the cells, as also shown by the X-Z cross sections from the Z-stack acquisitions (Fig. 6C). Therefore, A β 42^{MO} was a very useful tool to further investigate the role of $_{in}$ A β 42 in synaptic dysfunction. In autaptic microcultures exposed to extracellularly applied A β 42^{MO} ($_{ex}$ A β 42^{MO}, 200 nM), no significant changes in either evoked EPSC amplitudes (T_{20} vs T_0 ; $p = 0.10$; $n = 9$; Fig. 6D) or mEPSC amplitude and frequency ($p = 0.92$ and $p = 0.36$, respectively; $n = 7$; Fig. 6E,F) were

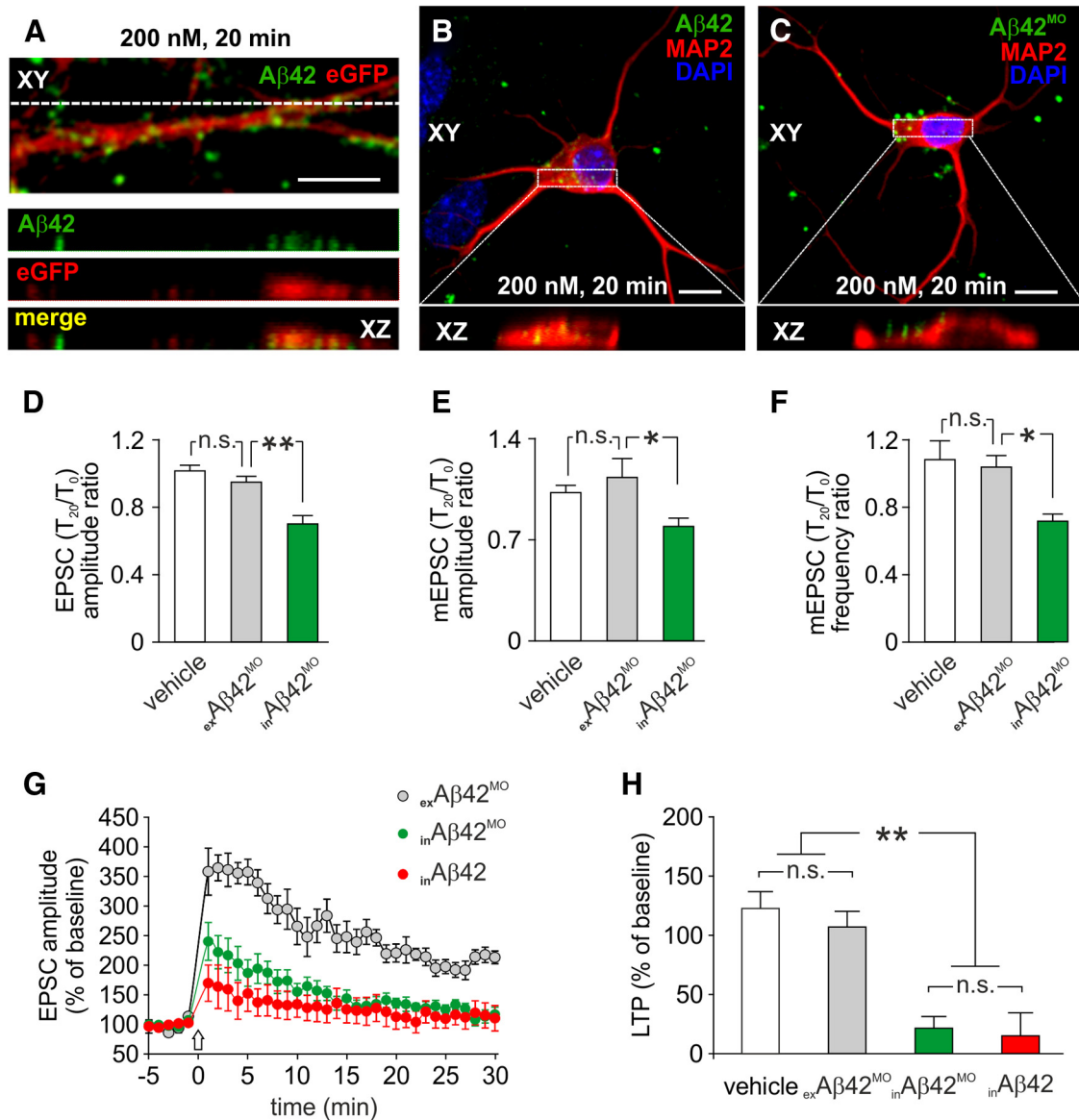


Figure 6. Extracellular A β 42^{MO} has no effects on synaptic transmission and plasticity but induces synaptic depression and LTP inhibition after patch-pipette dialysis. **A**, Representative example of a dendrite from eGFP⁺ hippocampal neuron (red) following 20 min extracellular application of 200 nM IRIS 5-labeled A β 42 (green). Representative examples of neurons exposed for 20 min to 200 nM IRIS 5-labeled A β 42 (**B**) and A β 42^{MO} (**C**). Red staining indicates MAP2 immunoreactivity. Bottom boxes in **A–C** represent X-Z cross sections from the Z-stack acquisitions showing the different neuronal accumulation of A β 42 analogs after 20 min treatments. Bar graphs comparing the T₂₀/T₀ ratio of EPSC amplitude (**D**), mEPSC amplitude (**E**), and mEPSC frequency (**F**) measured in autaptic neurons following application of vehicle (white bars), ex-A β 42^{MO} (gray bars), and in-A β 42^{MO} (green bars). **G**, Time course of EPSC amplitudes before and after HFS (indicated by arrow) in hippocampal slices treated with ex-A β 42^{MO} (gray circles), in-A β 42^{MO} (green circles), and in-A β 42 (red circles). **H**, Bar graphs (mean \pm SEM) showing the EPSC amplitudes measured during the last 5 min of recording under the conditions described for **G** and during the last 5 min of vehicle. Scale bars: **A–C**, 10 μ m. * p < 0.05; ** p < 0.005; n.s.: p > 0.05.

observed. Of note, 20 min after intracellular application of 200 nM A β 42^{MO} (in-A β 42^{MO}) both EPSC and mEPSC were significantly changed (EPSC: $-30.4 \pm 4.0\%$; $n = 17$; $p < 0.005$; mEPSC amplitude: $-20.7 \pm 5.7\%$; $n = 12$; $p < 0.05$; mEPSC frequency: $-28.4 \pm 3.9\%$; $n = 12$; $p < 0.05$; Fig. 6D–F). These findings indicate that intraneuronal uploading of soluble A β oligomers is critical for A β -mediated depression of basal synaptic transmission.

To determine whether intraneuronal accumulation was also required for A β 42-induced LTP inhibition, we examined the effects of ex-A β 42^{MO} and in-A β 42^{MO} in hippocampal brain slices. Extracellular application of 200 nM A β 42^{MO} had no effects on synaptic plasticity: LTP was $106.6 \pm 12.3\%$ ($n = 8$) and $119.7 \pm 17.8\%$ ($n = 8$), with ex-A β 42^{MO} and vehicle, respectively (Fig.

6G,H). On the contrary, this A β variant inhibited LTP to a similar level of wt in-A β 42 when applied via the patch pipette ($21.5 \pm 9.5\%$; $n = 10$; $p < 0.005$ vs vehicle; Fig. 6G,H).

Effects of ex-A β 42 on synaptic dysfunction likely depended on its ability to accumulate intraneuronally

To further investigate the role of intracellular accumulation of A β 42 and its internalization from extracellular space in the synaptic dysfunction, we tested the effects of ex-A β 42 on EPSCs, mEPSCs, and LTP after loading the recorded neurons with an antibody raised against human A β 42 (6E10; 1:300). This antibody recognizes the sequence 1–16 of human A β 42 (Tampellini et al., 2007) and, therefore, it counteracts the action of human synthetic A β 42 we used in our experiments. Injection of 6E10

into CA1 pyramidal neurons through the patch pipette ($_{in}6E10$) did not, per se, significantly affect LTP ($132.4 \pm 24.9\%$, $n = 9$), but it protected against the toxic effects of $_{ex}A\beta42$: LTP recorded with $_{ex}A\beta42 + _{in}6E10$ and $_{ex}A\beta42$ alone were $104.8 \pm 16.3\%$ ($n = 9$) and $66.2 \pm 12.6\%$ ($n = 8$; $p < 0.01$ vs vehicle), respectively ($_{ex}A\beta42 + _{in}6E10$ vs $_{ex}A\beta42$; $p < 0.05$; Fig. 7A, B). These findings clearly suggested that internalization of $_{ex}A\beta42$ is critical to LTP inhibition induced by A $\beta42$. We performed similar experiments in autaptic neurons and we found that $_{in}6E10$ counteracted the alterations of basal synaptic transmission induced by $_{ex}A\beta42$ in terms of both EPSC amplitude ($_{ex}A\beta42 + _{in}6E10$ vs $_{ex}A\beta42$; $p < 0.05$) and mEPSC frequency ($_{ex}A\beta42 + _{in}6E10$ vs $_{ex}A\beta42$; $p < 0.05$; $n = 15$; Fig. 7C–E).

Discussion

Intraneuronal accumulation of A β is emerging as a key determinant of AD pathogenesis because it has been proposed to play a critical role in synaptic dysfunction underlying the cognitive impairment observed in AD (Gouras et al., 2000, 2010, 2012; Billings et al., 2005; LaFerla et al., 2007; Moreno et al., 2009; Bayer and Wirths, 2010; Nomura et al., 2012; Eimer and Vassar, 2013). However, most of these studies relied on molecular findings. Functional studies demonstrated that in squid giant synapses intraneuronal A β reduced the rate of rise of EPSPs that, eventually, became subthreshold for action potential generation (Moreno et al., 2009). These effects were attributed to diminished docked synaptic vesicles in A $\beta42$ -microinjected terminals with no significant changes in the clathrin-coated vesicles (Nomura et al., 2012). However, the synaptic dysfunction caused by intraneuronal A β in glutamatergic mammalian neurons and the underlying mechanisms are far from being fully understood. Our study investigated the contribution of intracellular accumulation of A $\beta42$ to alterations of glutamatergic synaptic transmission and plasticity focusing on A $\beta42$ internalization as a critical event in its synaptotoxicity. In particular, our paper demonstrates that A $\beta42$ uploading from the extracellular space and its intracellular accumulation dramatically affect a number of functional synaptic parameters. A major advantage of our approach is that we tested the effects of an A $\beta42$ variant unable to cross plasma membranes and studied the effects of $_{ex}A\beta42$ after neuronal loading with an antibody raised against human A $\beta42$. Collectively, the results of these experiments allowed us to elucidate the role of $_{in}A\beta42$ in synaptic dysfunction. Of note, the intracellular application of A $\beta42$ through patch pipette bypassed and ruled out mechanisms and/or intracellular pathways activated by A β binding to membrane receptors.

Many literature reports have suggested that A $\beta42$ binding to membrane receptors affects molecular mechanisms governing synaptic function. A β -mediated synaptotoxicity was attributed to activation of the metabotropic glutamate receptors, mGluR5, and stimulation of three kinases, JNK1, Cdk5, and p38 MAPK,

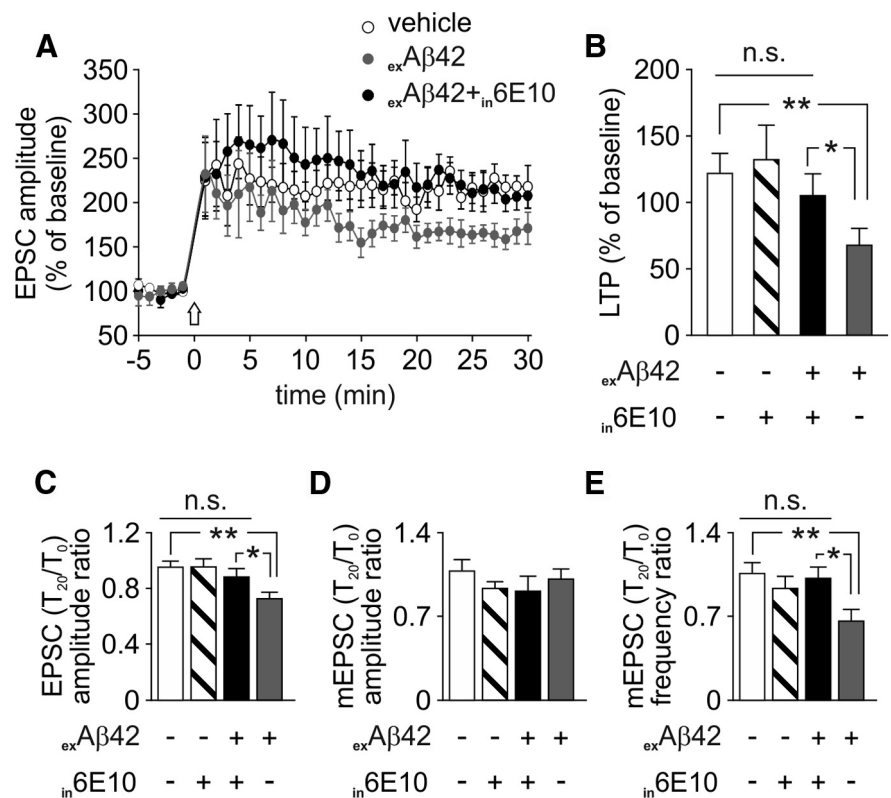


Figure 7. Inhibition of hippocampal LTP and basal synaptic transmission induced by $_{ex}A\beta42$ likely depends of its ability to be uploaded intraneuronally. **A**, Time course of EPSC amplitudes before and after HFS (indicated by arrow) in hippocampal slices treated with vehicle (white circles), $_{ex}A\beta42$ (gray circles), and $_{ex}A\beta42 + _{in}6E10$ (black circles). **B**, Bar graphs (mean \pm SEM) showing the EPSC amplitudes measured during the last 5 min of recording under the conditions described for **A** and during the last 5 min of $_{in}6E10$ alone. Bar graphs comparing the T_{20}/T_0 ratio of EPSC amplitude (**C**), mEPSC amplitude (**D**), and mEPSC frequency (**E**) measured in autaptic neurons following application of vehicle (white bars), $_{in}6E10$ (striped bars), $_{ex}A\beta42 + _{in}6E10$ (black bars), and $_{ex}A\beta42$ (gray bars). * $p < 0.05$; ** $p < 0.01$; n.s.: $p > 0.05$.

mediating LTP inhibition (Wang et al., 2004). Moreover, A β has been reported to interact with $\alpha7$ -containing nicotinic acetylcholine receptors (nAChRs; Wang et al., 2000; Dineley et al., 2001; Liu et al., 2001; Pettit et al., 2001). Glutamate release was reportedly affected by A β via inhibition of presynaptic nAChR (Dougherty et al., 2003). A β was also suggested to trigger NMDA receptor internalization following nAChR activation (Snyder et al., 2005). Moreover, A β binding to receptors for advanced glycation end products (RAGE) mediated MAPK phosphorylation leading to synaptic dysfunction (Origlia et al., 2009a,b). A β oligomers have also been reported to bind the fibronectin repeats domain of EphB2 and trigger EphB2 degradation causing deficits in learning and memory (Cissé et al., 2011). More recently, Kim et al. (2013) identified two new receptors for A β : the murine PirB and its human ortholog LILRB2 whose activation triggered a signaling cascade affecting the actin cytoskeleton and causing synaptic loss. Finally, experimental evidence suggests that APP itself is required for extracellular A β signaling (Lorenzo et al., 2000; Shaked et al., 2006).

Definitely, a number of molecular pathways activated by membrane receptors have been proposed to underlie A $\beta42$ -induced synaptic dysfunction. However, our findings allow us to hypothesize that synaptic dysfunction primarily depends on direct A $\beta42$ interaction with intracellular partners. In our view A $\beta42$ binding to membrane receptors might play a key role in A β internalization rather than directly triggering intracellular molecular pathways leading to synaptic dysfunction. Indeed, the

NMDA receptor antagonist AP5 completely blocked A β 42 internalization (Bi et al., 2002), which was facilitated by A β 42 binding to α 7-nAChRs, followed by endocytosis of the resulting complex (Nagele et al., 2002).

Our confocal microscopy experiments documented that $_{ex}$ A β 42 was rapidly internalized and accumulated at both somatic and dendritic levels. Hence, we asked whether the effects of $_{ex}$ A β 42 depended on its ability to cross the plasma membrane. We found that, in autaptic hippocampal neurons, 20 min of $_{ex}$ A β 42 selectively altered mEPSC frequency, i.e., the presynaptic vesicular release machinery (Ripoli et al., 2013), whereas $_{in}$ A β 42 affected both presynaptic and postsynaptic mechanisms. A beside effect of $_{ex}$ A β 42 was reported in APP-overexpressing neurons (Ting et al., 2007) and in autaptic microcultures exposed to $_{ex}$ A β 42 for longer (24–72 h) periods (Ripoli et al., 2013). We recently reported a time-dependent A β -induced alteration of glutamatergic synapses starting with changes in glutamate release and speculated that presynaptic alterations may represent the earliest step in synaptic dysfunction, followed by postsynaptic compartment impairment (Ripoli et al., 2013). Lipton's group recently confirmed A β -induced early synaptic injury consisting in decreased mEPSC frequency and suggested that the initial synaptic dysfunction, eventually followed by synapse loss, is due to excessive activation of extrasynaptic or perisynaptic NMDA receptors by glutamate released from astrocytes (Talantova et al., 2013). In our experimental model, the synaptic dysfunction caused by short-lasting A β 42 applications was not associated to neuronal damage/death or synaptic loss, as documented by the results of TUNEL assay and immunoreactivity for annexin V, synaptophysin, and PSD-95. Our data suggest a model of A β 42-induced synaptotoxicity including multiple presynaptic and postsynaptic mechanisms activated in a time-dependent manner. Of note, $_{ex}$ A β 42 has been shown to accumulate presynaptically and to directly compete with VAMP2 for binding to synaptophysin (Russell et al., 2012). Collectively, these results provide evidence that A β 42 internalization is critical to deregulation of glutamatergic synapses.

We also found that $_{in}$ A β 42 markedly inhibited LTP at CA3–CA1 synapses. The effects of $_{in}$ A β 42 on LTP were recently investigated by Nomura et al. (2012) who did not find LTP inhibitions at $_{in}$ A β 42 concentrations >1 nM. Instead, we found that 200 nM $_{in}$ A β 42 produced a marked LTP inhibition whose specificity was documented by the absence of effects when 200 nM A β 42-1 was injected into the studied neuron. A possible explanation of this discrepancy might depend on the different protocols we used for A β 42 preparations. Indeed, Nomura et al. (2012) dissolved A β 42 in DMSO and soon after they diluted it in the internal recording solution, whereas we induced A β oligomer formation by 12–18 h incubation at 4°C before experiments. These different protocols may markedly affect A β oligomerization as suggested by our mass spectrometry analyses revealing a prevalence of monomers when A β 42-DMSO solutions were diluted in saline and studied within few hours (data not shown).

The marked effects of $_{in}$ A β 42 on basal synaptic transmission and LTP suggest that A β 42 internalization is required to alter glutamatergic synapses. This conclusion was supported by the results of experiments performed with an A β variant, A β 42^{MO}, which we previously demonstrated to exhibit soluble small-oligomer distribution similar to that of *wt*A β but is unable (1) to dysregulate Ca²⁺ currents, (2) to activate caspase-3 and induce apoptosis, (3) to markedly affect the expression of synaptic proteins and synaptic function, and (4) to cross the cell membrane (Clementi et al., 2006; Piacentini et al., 2008a; Maiti et al., 2011;

Ripoli et al., 2013). Here we clearly demonstrated that the lack of synaptotoxic effects exhibited by A β 42^{MO} depends on its inability to be uploaded intraneuronally. Indeed, A β 42^{MO} does not affect synaptic transmission and plasticity when applied extracellularly but produces synaptic depression and LTP inhibition virtually identical to those caused by *wt*A β 42 when it is injected into neurons via the recording electrode.

Further support to our contention that A β 42 internalization and its intracellular accumulation are critical to A β 42-induced synaptotoxicity came from the findings that injection of 6E10 antibody into the studied neuron blocked $_{ex}$ A β 42-dependent alterations of basal synaptic transmission and LTP. These findings suggest that A β 42 synaptotoxicity occurs independently of intracellular pathways activated by A β 42 interaction with membrane receptors. Molecular mechanisms leading to alterations of synaptic transmission and plasticity might primarily depend on A β interaction with intracellular molecular targets. Further studies are required to identify the causative events behind A β 42 internalization and the intracellular interactors of A β 42 that are responsible for its presynaptic and postsynaptic effects. A systemic proteomic analysis of intracellular A β 42 partners will hopefully allow us to identify intraneuronal molecular targets useful to design novel drugs preventing and/or counteracting synaptic dysfunction and cognitive decline in AD.

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