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N-Methyl-D-aspartate receptors are required for synaptic targeting of Alzheimer's toxic amyloid-β peptide oligomers

Helena Decker,*,† Sofia Jürgensen,* Martin F. Adrover,‡ Jordano Brito-Moreira,* Theresa R. Bomfim,* William L. Klein,§ Alberto L. Epstein,¶ Fernanda G. De Felice,* Diana Jerusalinsky‡ and Sergio T. Ferreira*

*Institute of Medical Biochemistry, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

†Program in Morphological Sciences, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

‡Institute of Cellular Biology & Neuroscience 'Professor Eduardo de Robertis', School of Medicine & CBC, University of Buenos Aires & CONICET, Buenos Aires, Argentina

§Department of Neurobiology and Physiology, Northwestern University, Evanston, Illinois, USA

¶Centre de Génétique Moléculaire et Cellulaire, Université Claude Bernard, Lyon 1, Villeurbanne, CNRS, France

Abstract

Soluble amyloid-β peptide (Aβ) oligomers, known to accumulate in Alzheimer's disease brains, target excitatory post-synaptic terminals. This is thought to trigger synapse deterioration, a mechanism possibly underlying memory loss in early stage Alzheimer's disease. A major unknown is the identity of the receptor(s) targeted by oligomers at synapses. Because oligomers have been shown to interfere with *N*-methyl-D-aspartate receptor (NMDAR) function and trafficking, we hypothesized that NMDARs might be required for oligomer binding to synapses. An amplicon vector was used to knock-down NMDARs in mature hippocampal neurons in culture, yielding 90% reduction in dendritic NMDAR expression and blocking neuronal oxidative stress induced by Aβ oligomers, a pathological response that has been shown to be mediated by NMDARs. Remarkably, NMDAR knock-down abolished oligomer binding to dendrites, indicating that NMDARs are required for synaptic targeting of oligomers. Nevertheless, oligomers do not appear to bind directly to NMDARs as indicated by the fact that both oligomer-attacked and non-attacked neurons exhibit similar surface levels of NMDARs. Furthermore, pre-treatment of neurons with insulin down-regulates oligomer-binding sites in the absence of a parallel reduction in surface levels of NMDARs. Establishing that NMDARs are key components of the synaptic oligomer binding complex may illuminate the development of novel approaches to prevent synapse failure triggered by Aβ oligomers.

Keywords: Aβ oligomers, Alzheimer's disease, insulin, NMDA receptor, synapse dysfunction.

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N-Methyl-D-aspartate receptors (NMDARs) are critical for synaptic plasticity mechanisms underlying learning and memory, such as long-term potentiation (LTP) (Morris *et al.* 1986; Collingridge and Singer 1990; Bliss and Collingridge 1993). However, aberrant activation of NMDARs has been implicated in a number of pathological conditions, including the deleterious neuronal impact of soluble amyloid-β peptide (Aβ) oligomers (Snyder *et al.* 2005; De Felice *et al.* 2007; Lacor *et al.* 2007; Shankar *et al.* 2007; Decker *et al.* 2010). Aβ oligomers are neurotoxins that accumulate in the brains and CSF of Alzheimer's disease (AD) patients (Gong *et al.* 2003; Lacor *et al.* 2004; Georganopoulou *et al.* 2005), acting as gain-of-function pathogenic ligands that attack synapses (Klein *et al.* 2004; Walsh and Selkoe 2004) and block LTP (Walsh *et al.* 2002; Wang *et al.* 2002; Shankar *et al.* 2008). Recent studies have shown that binding of Aβ oligomers to neurons instigates AD-type tau hyperphosphorylation (De Felice *et al.* 2008), changes in synapse structure and receptor

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Address correspondence and reprint requests to Sergio T. Ferreira, Institute of Medical Biochemistry, Federal University of Rio de Janeiro, Rio de Janeiro, RJ 21944-590, Brazil. E-mail: ferreira@bioqmed.ufrj.br

Abbreviations used: Aβ, amyloid-β peptide; AD, Alzheimer's disease; AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; DHE, dihydroethidium; DIV, days *in vitro*; DMSO, dimethyl sulfoxide; IR, insulin receptor; LTP, long-term potentiation; NMDA, *N*-methyl-D-aspartate; NMDAR, NMDA subtype glutamate receptor; $p75^{NTR}$, p75 neurotrophin receptor; PBS, phosphate-buffered saline; PrP^{C}, cellular prion protein; ROS, reactive oxygen species.

composition (Lacor *et al.* 2007; De Felice *et al.* 2009), oxidative stress (De Felice *et al.* 2007) and impairment of fast axonal transport (Decker *et al.* 2010). The attack on synapses by Aβ oligomers thus provides a unifying mechanism linking major features of AD neuropathology with the characteristic memory dysfunction in AD (Klein 2006; Ferreira *et al.* 2007).

Amyloid-β oligomers cause deregulation of NMDAR function, initially disrupting calcium homeostasis and triggering neuronal oxidative stress (Kelly and Ferreira 2006; De Felice *et al.* 2007), followed by removal of NMDARs from dendrites (Snyder *et al.* 2005; Lacor *et al.* 2007; Shankar *et al.* 2007; De Felice *et al.* 2009). These responses are thought to contribute to plasticity failure and memory dysfunction in AD. We recently found that antibodies against the extracellular domain of the NR1 subunit of NMDARs markedly reduce Aβ oligomer binding to neurons (De Felice *et al.* 2007). This suggests that Aβ oligomers bind to synapses in close proximity to NMDARs, raising the possibility that NMDARs are necessary components of or are required for the assembly of the receptor complex that binds oligomers.

In the present study, we have used a herpes virus-derived amplicon vector expressing an antisense sequence for the NR1 subunit (Adrover *et al.* 2003; Cheli *et al.* 2006) to knock-down the expression of NMDARs in cultured hippocampal neurons. Results showed that although control neurons attacked by Aβ oligomers exhibit abnormally elevated reactive oxygen species (ROS) levels, this pathological response was abolished in NMDAR knock-down neurons. Significantly, knock-down of NMDARs abolished the binding of Aβ oligomers to dendrites, suggesting that NMDARs might be the targets of oligomer binding. Additional results, however, indicated that, although required for dendritic targeting of Aβ oligomers, NMDARs are likely not the molecules to which oligomers directly bind. Identifying the molecular targets of Aβ oligomers may pave the way for development of new approaches to prevent oligomer binding to synapses, effectively halting the progression of AD.

Material and methods

Materials

$A\beta_{1–42}$ peptide was from American Peptides (Sunnyvale, CA, USA). Insulin, 1,1,1,3,3,3,-hexafluoro-2-propanol, dimethyl sulfoxide (DMSO) and poly-L-lysine were from Sigma (St Louis, MO, USA). Culture media/supplements, Alexa-labeled secondary antibodies, ProLong and dihydroethidium (DHE) were from Invitrogen (Carlsbad, CA, USA). BCA protein assay kit was from Pierce (Deerfield, IL, USA). Antibody against the NR1 subunit of NMDARs was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Neuronal cultures

Primary cultures of hippocampal neurons from E18 rat embryos were prepared and maintained in Neurobasal medium supplemented with B27 (Invitrogen) for 18 days as previously described (Paula-Lima *et al.* 2005; De Felice *et al.* 2007).

Viral vector construction

Vectors were prepared using amplicon plasmids expressing the open reading frame of enhanced green fluorescent protein and either the cDNA of NR1 in antisense (−)orientation [pA-GFP-NR1(−)] or the LacZ gene (pA-GFP-LacZ) (Adrover *et al.* 2003). Briefly, 7b cells (Krisky *et al.* 1998) were transfected with either amplicon plasmid using Lipofectamine Plus (Invitrogen; Paisley, UK). One day later, cells were infected with the LaLΔJ helper virus at a multiplicity of infection of 0.3 p.f.u. (plaque forming unit) per cell. When the cytopathic effect was maximal, cells were collected by centrifugation, disrupted by three cycles of freeze/ thawing to release vector particles and centrifuged at 5000 *g* for 5 min to pellet cell debris. The supernatant was collected and stored at −80°C. Herpes simplex virus-1 LaLΔJ helper virus particles were titrated by plaque assay on 7b cells (Machuca *et al.* 1986) and on Vero cells for identification of potential replication-competent revertant particles. To titrate vector particles, Gli36 cells were infected with serial dilutions of each vector stock and cells expressing enhanced green fluorescent protein were scored using a Zeiss Axiophot microscope, as described (Zaupa *et al.* 2003). Titers of all amplicon vector stocks used in this study were above 10^8 particles/mL.

Preparation and characterization of Aβ oligomers

Aβ oligomers (also known as Abeta-derived diffusible ligands, ADDLs) were prepared as previously described (Lambert *et al.* 1998; Gong *et al.* 2003). Briefly, $A\beta_{1\text{-}42}$ (American Peptides, Sunnyvale, CA, USA) was dissolved in cold hexafluoro-2-propanol (Merck, Darmstadt, Germany), incubated at 23°C for 60 min plus 10 min on ice and the solution was allowed to evaporate. The dried films were stored at −80°C. For oligomer preparation, films were resuspended to 5 mM Aβ in anhydrous DMSO (Sigma), diluted to 100 μM in cold sterile phosphate-buffered saline (PBS) and incubated at 4°C for 24 h. The preparation was centrifuged at 14 000 *g* for 10 min at 4°C to remove insoluble aggregates and the supernatant containing soluble Aβ oligomers was transferred to clean tubes and stored at 4°C. Oligomer solutions were used within 24 h of preparation. Characterization of oligomer preparations was routinely performed by size exclusion chromatography on a GPC-100 column (Eprogen, Darien, IL, USA) and, occasionally, by transmission electron microscopy and western immunoblot probed with rabbit polyclonal anti-oligomer antibody (Figure S1). As shown in Figure S1, oligomers ranged from dimers (∼9 kDa) to higher molecular weight oligomers (∼50–100 kDa). Protein concentration was determined using the BCA Protein assay (Pierce, Rockford, IL, USA) and bovine serum albumin as a standard.

Experimental treatments of cultures and immunocytochemistry

Hippocampal neurons [18 days *in vitro* (DIV)] were exposed to A-GFP-NR1(−) or A-GFP-LacZ (mock) vectors for 2 h at 37°C (in a 5% CO_2 atmosphere). Vector-containing medium was removed and cells were incubated for 30 hours at 37°C in conditioned medium produced by the same culture. Cultures were immunolabeled for NMDAR (anti-NR1; Santa Cruz, Temecula, CA, USA; 1 : 50 dilution) to determine the efficiency of knock-down (relative

to control non-infected or LacZ-infected cultures). Additional infected cultures were treated for different times with Aβ oligomers or vehicle, as indicated under Results section. Insulin (1 μM), when present, was added to non-infected neurons 30 min before addition of Aβ oligomers. Immunocytochemistry was performed as previously described (De Felice *et al.* 2008) using anti-NR1 or monoclonal Aβ oligomer-selective NU4 antibody (Lambert *et al.* 2007) (1 : 1000 dilution) and anti-rabbit or anti-mouse Alexa Fluor 555-conjugated secondary antibody, respectively (1 : 1000 dilution; Molecular Probes, Eugene, OR, USA). Coverslips were mounted using Prolong Gold (Molecular Probes). Cells were imaged on a Nikon Eclipse TE300 microscope or on a Zeiss LSM510 META confocal microscope**.** Quantitative analysis of immunofluorescence data was carried out using NIH Image J (Windows version) as described (De Felice *et al.* 2008).

Neuronal oxidative stress

Formation of ROS was measured in live neurons using DHE (Molecular Probes), a fluorescent probe sensitive to various ROS species, including hydrogen peroxide, hydroxyl radical, peroxyl radicals and peroxynitrite. Cultures incubated for 5 h at 37°C with 1 μM Aβ oligomers or vehicle were loaded with DHE (10 μM) during the last 40 min of incubation with oligomers. Cells were rinsed three times with warm PBS, once with phenol red-free Neurobasal medium and were immediately imaged on the Nikon microscope. Analysis of DHE fluorescence was carried out using Image J as described (De Felice *et al.* 2007). Appropriate thresholding was employed to eliminate background signal before histogram analysis. In each experiment, 16 images were analyzed per experimental condition to allow quantitative determination of changes in neuronal ROS levels. At least three experiments using independent neuronal cultures were performed and integrated fluorescence levels were normalized by the number of cells in each image.

Data analysis

Quantitative analysis of immunofluorescence data was carried out using NIH Image J (Windows version) as described (De Felice *et al.* 2007). Appropriate thresholding was employed to eliminate background signal before histogram analysis. Cell bodies were digitally removed from the images so that only dendrite immunofluorescence was analyzed. In each experiment, twenty four images were analyzed per experimental condition to allow quantitative determination of changes in NMDAR or Aβ oligomer immunofluorescence levels. At least three experiments using independent cultures were performed and results were normalized by the number of cells in each image. NR1 or Aβ immunofluorescence levels in individual dendrites imaged by confocal microscopy were determined by histogram analysis using Image J in 12 individual dendrite segments (20 μm each).

Results

NMDAR knock-down in hippocampal neurons

To investigate the involvement of NMDARs in Aβ oligomer binding, we initially aimed to knock-down NMDARs in mature hippocampal neurons in culture. Expression of NMDARs was disrupted by infection with an amplicon vector expressing an antisense transgene directed to the constitutive NR1 subunit of NMDARs. This vector has been previously shown to cause a major decrease in NR1 protein levels both in primary neurons in culture and in the hippocampi of adult rats (Adrover *et al.* 2003; Cheli *et al.* 2006). Neuronal cultures (18 DIV) were infected by either A-GFP-NR1(−) (NR1 antisense vector) or A-GFP-LacZ (control vector for mock infection) at a multiplicity of infection of 5. About 40% of the neurons were infected (as determined by GFP expression; Figure S2A) and cell viability was preserved in infected cultures (Figure S2B). Neurons infected by A-GFP-LacZ (mock infection) exhibited punctate dendritic labeling for NR1, indistinguishable from the pattern exhibited by non-infected control neurons (Fig. 1a and c). By contrast, overall NR1 immunoreactivity was markedly reduced in neurons infected by A-GFP-NR1(−) (Fig. 1b and j) compared to mock-infected controls. Image analysis of individual dendrite segments revealed ∼90% decrease in dendritic NMDAR expression in A-GFP-NR1(−)-infected neurons compared with mock-infected neurons (Fig. 1h, I and k). These results demonstrate the efficiency of NMDAR knock-down in our cultures.

Aβ oligomer-induced oxidative stress is blocked in NMDAR knock-down neurons

Aβ oligomers have been shown to instigate excessive neuronal ROS formation through a mechanism mediated by NMDARs (De Felice *et al.* 2007). To confirm that the function of NMDARs was indeed blocked in knock-down neurons, we investigated whether oxidative stress induced by AβOs was abolished in those neurons. In line with our expectation, Aβ oligomer-induced ROS formation was evident in non-infected neurons but it was blocked specifically in NMDAR knock-down neurons (Fig. 2).

NMDAR knock-down eliminates binding of Aβ oligomers

We have recently shown that antibodies against the extracellular N-terminal domain of the NR1 subunit of NMDARs partially (∼60%) blocked binding of Aβ oligomers to neurons in culture (De Felice *et al.* 2007). 2-Amino-5-phosphonopentanoic acid (D-AP5), an NMDAR antagonist, also caused a reduction in binding, suggesting that oligomers bind either directly to NMDARs or to a protein that interacts closely with NMDARs (De Felice *et al.* 2007). To determine whether NMDARs are required for Aβ oligomer targeting to neurons, we investigated oligomer binding to NMDAR knock-down neurons. Remarkably, A-GFP-NR1(−)-infected neurons showed virtually no binding of Aβ oligomers to their processes (Fig. 3b, h and i). In contrast, mock-infected neurons exhibited a characteristic punctate pattern of dendritic Aβ oligomer binding similar to non-infected neurons (Figure 3a and c), ruling out the possibility that the reduced binding in knock-down neurons could be a non-

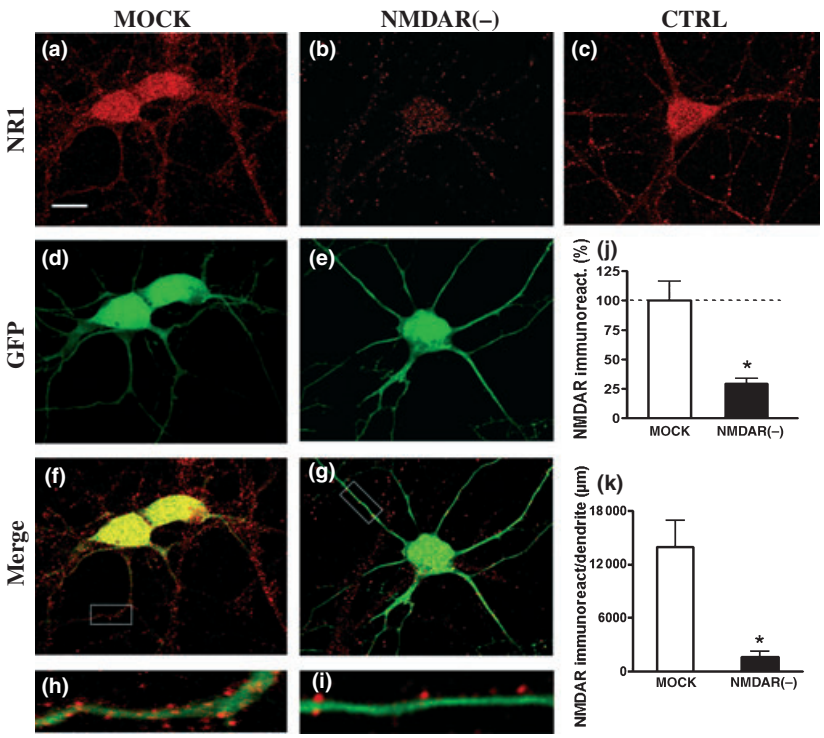

Fig. 1 NMDAR knock-down in mature hippocampal neurons. Cultured hippocampal neurons (18 DIV) were exposed to either A-GFP-NR1(–) vector [NR1 knock-down; indicated as NMDAR(–)] or A-GFP-LacZ vector (infection control; indicated as MOCK) followed by immunolabeling with anti-NMDAR (NR1 subunit). (a, c) Representative images from mock-infected or non-infected (CTRL) neurons; no differences in NR1 expression levels were detected between these conditions. (b) Representative image from an NMDAR knock-down [NMDAR(–)] neuron showing markedly reduced dendritic NR1 expression. (d, e) GFP fluorescence images of the same fields as in panels (a) and (b), respectively. (f, g) Overlays of NR1 immunofluorescence (red) and GFP fluorescence (green). (h, i) High-magnification images of dendrite segments contained in the dotted rectangles indicated in panels (f) and (g). (j, k) Quantification of NR1 levels based on integration of dendritic immunofluorescence in ~120 neurons (j) and in 12 individual (20 μm) dendrite segments (k). See also Figure S1. Statistically significant ($^{*}p < 0.001$ or $^{\#}p < 0.0001$, respectively) decreases compared with mock-infected neurons. Scale bar, 10 μm.

specific consequence of infection by the amplicon vector. Image analysis of individual dendrite segments revealed ~90% decrease in oligomer binding to NMDAR knock-down neurons (Fig. 3k), in excellent agreement with the reduction in dendritic NR1 immunoreactivity described above. These results show that oligomer binding to neurons requires the presence of surface NMDARs.

NMDARs are necessary but not sufficient for Aβ oligomer binding

Results described above raised the possibility that Aβ oligomers might bind directly to NMDARs. Previous studies have shown that Aβ oligomers target a subset of neurons typically comprising 50–70% of the total in mature hippocampal neuronal cultures (Lacor *et al.* 2004; Zhao *et al.* 2008; Decker *et al.* 2010). Moreover, Aβ oligomers have been shown to bind specifically to PSD-95-positive, glutamatergic terminals (Lacor *et al.* 2004, 2007). In harmony with these previous findings, oligomers bound only to a subset of hippocampal neurons in culture (Fig. 4d). Significantly, oligomer-attacked and non-attacked neurons exhibited similar levels of surface NMDAR immunoreactivity (Fig. 4b and e), suggesting that additional protein components are necessary for assembly of a receptor complex that binds Aβ oligomers at synapses.

To further investigate this issue, we sought an independent way to down-regulate oligomer binding without interference with neuronal NMDAR levels. To this end, we took advantage of our recent finding that insulin signaling down-regulates Aβ oligomer-binding sites at the neuronal surface (De Felice *et al.* 2009). Treatment of hippocampal neuronal cultures with insulin caused a significant (~50%) reduction in dendritic oligomer binding (Fig. 5e). If Aβ oligomers were indeed binding to NMDARs, one would expect to observe a parallel decrease in dendritic NMDAR immunoreactivity in insulin-treated neurons. In contrast, we found that insulin treatment did not induce down-regulation of NMDARs, but rather prevented the internalization of NMDARs that is induced by Aβ oligomers (Snyder *et al.* 2005; Lacor *et al.* 2007; De Felice *et al.* 2009). Thus, in the presence of insulin,

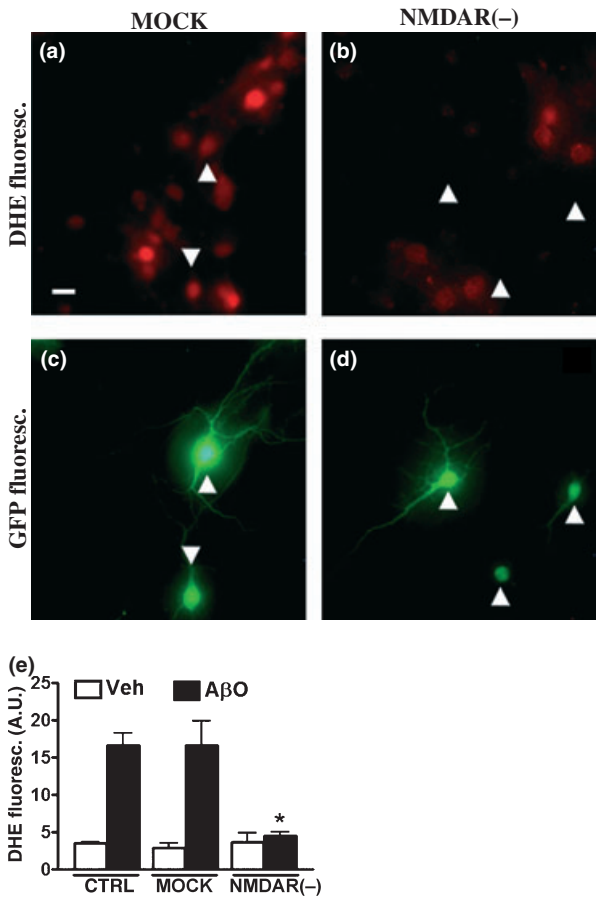

Fig. 2 ROS formation induced by Aβ oligomers (AβOs) is blocked in NMDAR knock-down neurons. Cultured hippocampal neurons (18 DIV) were exposed to either A-GFP-NR1[–) vector (NMDAR(–)] or A-GFP-LacZ vector (MOCK). After infection, neurons were incubated for 5 h at 37°C with 1 μM AβOs or vehicle. They were loaded with DHE (10 μM) during the last 40 min of incubation with oligomers. (a, b) Representative DHE fluorescence images from mock-infected or NMDAR(–) neurons, respectively, treated with AβOs. (d, e) GFP fluorescence images of the same fields as in panels (a) and (b), respectively. Similar to non-infected neurons, a prominent increase in ROS production induced by Aβ oligomers is evident in mock-infected neurons (a and c, arrowheads). In contrast, very low DHE fluorescence is observed in NMDAR(–) neurons (b and d, arrowheads). (e) Quantification of DHE fluorescence (see Methods section). Statistically significant (*$p < 0.001$) decrease compared with both mock-infected and non-infected neurons treated with AβOs. Scale bar, 40 μm.

Aβ oligomer binding is markedly reduced in the absence of any reduction in NMDAR levels at the neuronal membrane. Taken together, these results indicate that NMDARs are necessary but not sufficient for Aβ oligomer binding.

Discussion

Progress in AD research has been hampered by two fundamental issues with broad implications for our understanding of the molecular mechanisms of pathogenesis and for the rational development of novel and effective therapeutics. The first problem comprises the nature of the toxic species responsible for the initial memory impairment and for neuronal dysfunction in AD. In this regard, considerable advance has been made in the past decade with increased recognition that soluble Aβ oligomers are the proximal neurotoxins that attack synapses and cause synapse failure in the early phases of pathology. The other major unknown is the molecular identity of the receptor(s) that bind Aβ oligomers with high specificity at excitatory synapses. Identification of Aβ oligomer-binding receptor(s) may open the way for development of novel approaches to prevent neuronal targeting of Aβ oligomers.

Aβ oligomers are ligands for post-synaptic spines and co-localize with PSD-95 (Lacor *et al.* 2004). The specific binding of Aβ oligomers to synapses is lost upon controlled trypsin treatment of neurons, suggesting the involvement of cell-surface proteins (Lambert *et al.* 1998). A number of candidate oligomer-binding proteins have been proposed in the past few years. For example, an interesting recent study showed that Aβ oligomers bind with nanomolar affinity to cellular prion protein (PrP^C) and that anti-PrP antibodies reduce oligomer binding to neurons and rescue synaptic plasticity (Lauren *et al.* 2009). These findings suggest that PrP^C is involved in synaptic binding and mediates synaptotoxicity of Aβ oligomers (Gimbel *et al.* 2010). However, other studies have shown that PrP-expressing and PrP-knockout mice are equally susceptible to long-term memory impairment induced by Aβ oligomers (Balducci *et al.* 2010; Calella *et al.* 2010), supporting the notion that, at least in part, the deleterious effects of Aβ oligomers on plasticity are independent of PrP^C (Kessels *et al.* 2010).

Co-immunoprecipitation and photoactivated amino acid cross-linking studies indicated that Aβ oligomers interact with complexes containing the GluR2 subunit of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors, a conclusion that was corroborated by the observation that pharmacological inhibition or removal of surface AMPA receptors reduced Aβ oligomer binding to neurons (Zhao *et al.* 2010). Moreover, using single-particle tracking of quantum dot-labeled Aβ oligomers, a very recent study has demonstrated the participation of metabotropic glutamate receptors (mGluR5) in Aβ oligomer binding and clustering at synapses (Renner *et al.* 2010).

In addition, interaction of Aβ oligomers with the extracellular domain of p75 neurotrophin receptor ($p75^{NTR}$) has been reported to mediate Aβ oligomer-induced neuritic dystrophy *in vitro* and *in vivo* (Knowles *et al.* 2009). Other putative receptors involved in neuronal binding of Aβ oligomers include the receptor for advanced glycation end products (Sturchler *et al.* 2008), nicotinic acetylcholine receptors (Magdesian *et al.* 2005) and Frizzled (Magdesian *et al.* 2008), which leads to inhibition of Wnt/β-catenin signaling.

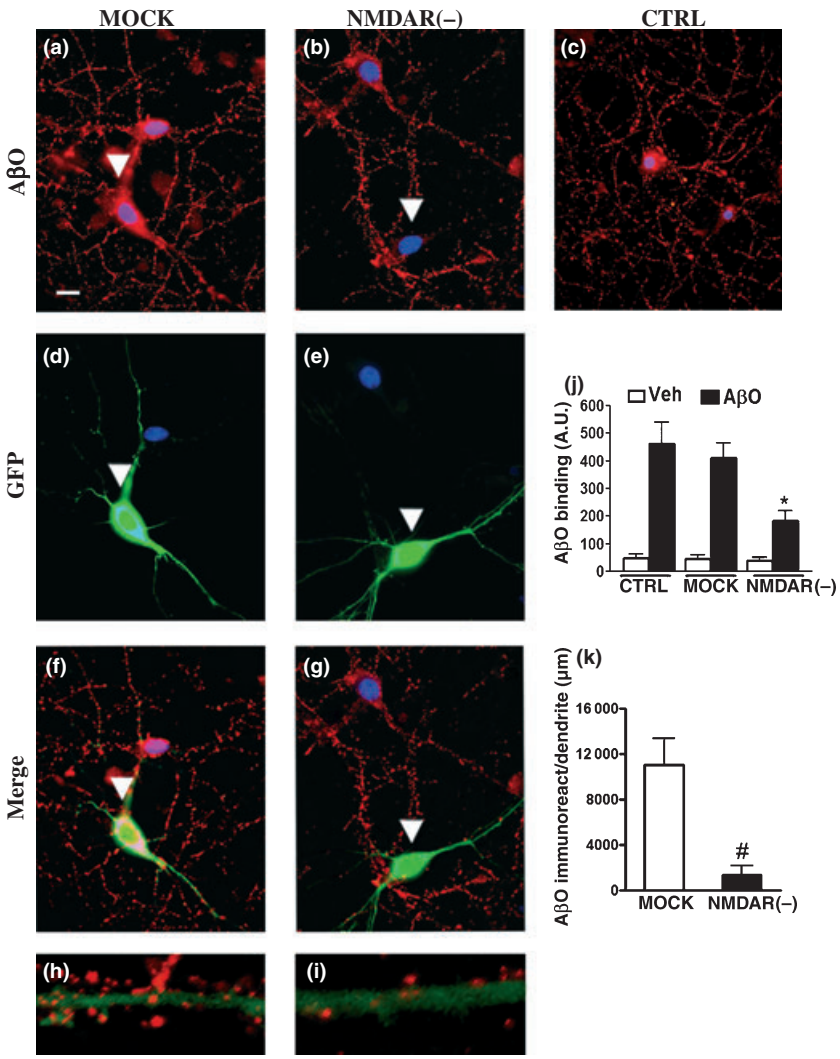

Fig. 3 Synaptic Aβ oligomer (AβO) binding is blocked in NMDAR knock-down neurons. Cultured hippocampal neurons (18 DIV) were exposed to either A-GFP-NR1(–) vector [NMDAR(–)] or A-GFP-LacZ vector (MOCK). After infection, neurons were treated for 15 min at 37°C with 500 nM AβOs or an equivalent volume of vehicle followed by immunolabeling using NU4 anti-oligomer antibody (Lambert *et al.* 2007). (a–c) Representative images from mock-infected, NMDAR(–) and non-infected (CTRL) neurons, respectively. (d, e) GFP fluorescence images of the same fields as in panels (a) and (b), respectively. Nuclear staining (DAPI) is shown in blue. Arrowheads point to cell bodies that were infected by A-GFP-NR1(–) or mock vectors. (f, g) Overlays of AβO immunofluorescence (red) and GFP fluorescence (green) in mock-infected or NMDAR(–) neurons, respectively. (h, i) High-magnification confocal images of dendrite segments from mock-infected or NMDAR(–) neurons, respectively. (j, k) Quantification of AβO immunofluorescence based on integration of dendritic immunofluorescence in ~120 neurons (j) and in 12 individual (20 μm) dendrite segments (k). Statistically significant ($^{*}p < 0.01$ and $^{\#}p < 0.001$, respectively) decreases compared with mock-infected neurons. Scale bar, 10 μm.

Until now, however, the identity of the specific receptor(s) that bind oligomers at synapses has remained elusive. Current results establish that NMDARs are required for oligomer binding to dendrites. We have previously demonstrated that NMDARs co-immunoprecipitate with Aβ oligomers from detergent-extracted oligomer-treated rat synaptosomal membranes and that an N-terminal anti-NR1 antibody markedly reduced oligomer binding to dendrites (De Felice *et al.* 2007). Consistent with those findings, we now show that oligomer binding is virtually abolished in dendrites of NMDAR knock-down neurons. One possible explanation for this result could be that Aβ oligomers bind directly to NMDARs at synapses. Interestingly, however, we found that similar NMDAR levels are found in both oligomer-attacked and non-attacked neurons, suggesting that presence of NMDARs alone is not sufficient for oligomer binding.

To further address the question whether Aβ oligomers directly bind NMDARs, we took advantage of our previous finding that insulin signaling down-regulates Aβ oligomer-binding sites (De Felice *et al.* 2009). Insulin plays a key role in plasticity mechanisms in the CNS (Zhao *et al.* 1999; Zhao and Alkon 2001) and has been shown to improve cognitive performance in patients with early stage AD (Reger *et al.* 2008). Insulin acts through the insulin receptor (IR), a protein tyrosine kinase with pivotal roles in regulation of peripheral glucose metabolism and energy homeostasis. IRs also occur in the brain, where they are abundantly distributed in synaptic membranes of the cerebral cortex and hippocampus (Heidenreich *et al.* 1983, 1988; Matsumoto and Rhoads 1990; Zhao *et al.* 1999). Of relevance to the current study, we have recently shown that insulin blocks Aβ oligomer-binding to synapses (De Felice *et al.* 2009). Decreased binding is the result of down-regulation of Aβ oligomer-binding sites by insulin, through a mechanism requiring IR tyrosine kinase activity.

Based on these previous findings, we have employed insulin as a tool to down-regulate Aβ oligomer-binding sites in hippocampal neurons and to examine the possibility

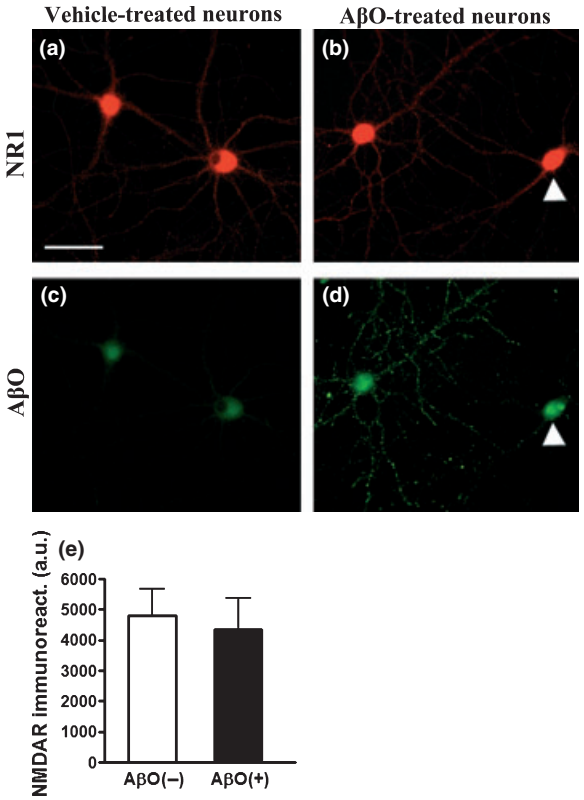

Fig. 4 Surface NMDAR levels are similar in Aβ oligomer-attacked and non-attacked neurons. Cultured hippocampal neurons (18 DIV) were treated for 15 min at 37°C with 500 nM Aβ oligomers (AβOs) or an equivalent volume of vehicle followed by double immunolabeling using anti-NMDAR (NR1 subunit, green) and NU4 anti-oligomer antibody (Lambert *et al.* 2007; red). Note that Aβ oligomer-attacked and non-attacked (arrowhead, d) neurons exhibit similar levels of NMDAR labeling (b). Scale bar, 50 μm. (e) Graph showing quantitative analysis of NR1 immunofluorescence in AβO-attacked [AβO(+), black bar] and non-attacked [AβO(–), white bar] neurons. Bars show integrated dendritic immunofluorescence levels from 12 neurons in each group, and correspond to mean ± SEM of two independent experiments carried out in triplicate each.

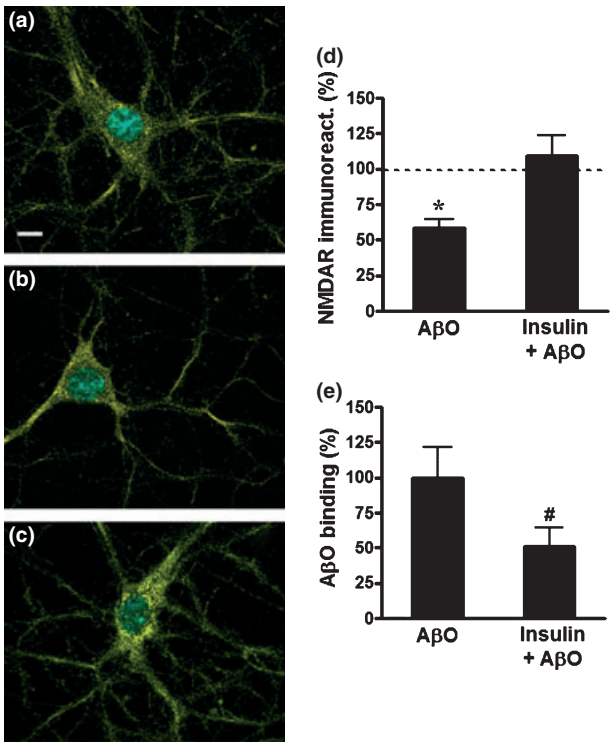

Fig. 5 Insulin blocks Aβ oligomer (AβO) binding but does not affect surface NMDAR levels. Cultures were pre-incubated with 1 μM insulin for 30 min and treated for 4 h at 37°C with 400 nM AβOs (or an equivalent volume of vehicle) followed by immunolabeling using anti-NMDAR (NR1 subunit) or NU4 anti-oligomer antibody. (a–c) Representative images of vehicle-treated, AβO-treated and (insulin + AβO)-treated cells, respectively, immunolabeled against NMDAR (yellow) and stained with DAPI (blue). Quantification of NR1 (d) or AβO immunofluorescence (e) was based on integration of dendritic immunofluorescence in ~90 neurons. Bars represent mean ± SEM of four independent experiments carried out in triplicate each. Statistically significant difference ($^{*}p < 0.05$) compared with vehicle-treated neurons; statistically significant difference ($^{\#}p < 0.05$) compared with AβO-treated neurons. Scale bar, 10 μm.

that NMDARs might be down-regulated in parallel, as one would expect if NMDARs were the oligomer-binding protein at synapses. Interestingly, pre-treatment of hippocampal neurons with insulin caused a significant reduction in oligomer binding in the absence of a parallel reduction in NMDAR levels. This indicates that NMDARs are not the oligomer binding receptor that is down-regulated by insulin. We thus conclude that, although required for binding of Aβ oligomers, NMDARs are not sufficient for binding. Results suggest that NMDARs are either constituents of a multi-protein receptor complex that binds Aβ oligomers (which may include other protein components, such as those mentioned above) or they are required for the assembly/surface expression of the Aβ oligomer-binding receptor.

Recent studies have shown that Aβ oligomers disrupt neuronal calcium homeostasis via aberrant activation of NMDARs (Kelly and Ferreira 2006; De Felice *et al.* 2007). NMDARs have also been implicated in other neuronal pathologies induced by oligomers, for example, tau hyperphosphorylation (De Felice *et al.* 2008), synapse loss (Lacor *et al.* 2007) and impairment of fast axonal transport of organelles (Decker *et al.* 2010). In a previous study, we investigated the connection between Aβ oligomer binding, NMDAR dysregulation and NMDAR-dependent neuronal oxidative stress (De Felice *et al.* 2007). In that study, we showed that memantine, a moderate affinity NMDAR blocker, potently inhibited Aβ oligomer-induced formation of reactive oxygen species (ROS) in hippocampal neurons. Similarly, MK-801, a higher affinity NMDAR blocker, and

D-AP5, a competitive antagonist, completely blocked Aβ oligomer-induced ROS formation. Importantly, however, memantine and MK-801 had no effect on Aβ oligomer binding to hippocampal neurons, and D-AP5 caused partial reduction in binding. We also investigated the effect of the AMPA receptor antagonist, 6,7-Dinitroquinoxaline-2,3-dione, on binding and toxicity of Aβ oligomers. Treatment of hippocampal cultures with 6,7-Dinitroquinoxaline-2,3-dione caused only a slight inhibition of ROS formation and had no effect on Aβ oligomer binding (De Felice *et al.* 2007). Together with the current results, these previous findings indicate that surface expression of NMDARs (but not necessarily their activity) is required for Aβ oligomer binding, and that dysregulated NMDAR activity triggers pathways leading to oligomer-induced neuronal dysfunction and toxicity.

We now show that oligomers failed to induce oxidative stress in NMDAR knock-down neurons, whereas non-infected or mock-infected neurons exhibited robust ROS response to oligomers. These observations support the idea that, in addition to their role in oligomer binding, NMDARs also mediate aberrant signaling that culminates in neuronal damage in AD.

NMDAR function is essential for learning, memory and for the induction of LTP (Morris 2001; Nakazawa *et al.* 2004; Lau and Zukin 2007). Moreover, specific subunits of NMDARs, such as NR2B, mediate synaptic plasticity *in vivo* (Hu *et al.* 2009; Ronicke *et al.* 2010). Thus, new drugs optimized as blockers of Aβ oligomer binding that do not impair physiological NMDAR function potentially could provide improved AD therapeutics. In this regard, we recently showed that antibodies blocking the N-terminal extracellular domain of NMDARs significantly reduced Aβ oligomer binding to neurons without interference with receptor response to glutamate (De Felice *et al.* 2007).

In conclusion, current results demonstrate that NMDARs play a pivotal role in the assembly and/or expression of the neuronal receptor that binds Aβ oligomers. This discovery supports the new concept of a multi-component assembly required for stabilizing the toxic accumulation of Aβ oligomers at the synaptic membrane. Whether oligomers target a pre-assembled receptor complex that already exists at the membrane or whether they act as scaffolding molecules to promote the assembly of such a complex (Renner *et al.* 2010) remains to the determined. Whatever the case, a key concept that derives from this notion is that clustering of Aβ oligomer-bound receptors may be required for toxicity, for example, by triggering excessive calcium influx and ROS formation. From a therapeutic point of view, this suggests the possibility that clustering and toxicity could be blocked by interfering with different molecular components of the receptor complex. Uncovering the identity of the synaptic targets of Aβ oligomers may provide insight into the complexity of the mechanisms that underlie synapse failure in AD and may pave the way for development of novel and more effective AD therapeutics.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1. Characterization of AβO preparations.

Figure S2. Knock-down efficiency and cell viability.

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