Aβ Oligomers Induce Neuronal Oxidative Stress through an N-Methyl-D-aspartate Receptor-dependent Mechanism That Is Blocked by the Alzheimer Drug Memantine*

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Oxidative stress is a major aspect of Alzheimer disease (AD) pathology. We have investigated the relationship between oxidative stress and neuronal binding of Aβ oligomers (also known as ADDLs). ADDLs are known to accumulate in brain tissue of AD patients and are considered centrally related to pathogenesis. Using hippocampal neuronal cultures, we found that ADDLs stimulated excessive formation of reactive oxygen species (ROS) through a mechanism requiring N-methyl-D-aspartate receptor (NMDA-R) activation. ADDL binding to neurons was reduced and ROS formation was completely blocked by an antibody to the extracellular domain of the NR1 subunit of NMDA-Rs. In harmony with a steric inhibition of ADDL binding by NR1 antibodies, ADDLs that were bound to detergent-extracted synaptosomal membranes co-immunoprecipitated with NMDA-R subunits. The NR1 antibody did not affect ROS formation induced by NMDA, showing that NMDA-Rs themselves remained functional. Memantine, an open channel NMDA-R antagonist preserved as a memory-preserving drug for AD patients, completely protected against ADDL-induced ROS formation, as did other NMDA-R antagonists. Memantine and the anti-NR1 antibody also attenuated a rapid ADDL-induced increase in intraneuronal calcium, which was essential for stimulated ROS formation. These results show that ADDLs bind to or in close proximity to NMDA-Rs, triggering neuronal damage through NMDA-R-dependent calcium flux. This response provides a pathologically specific mechanism for the therapeutic action of memantine, indicates a role for ROS dysregulation in ADDL-induced cognitive impairment, and supports the unifying hypothesis that ADDLs play a central role in AD pathogenesis.

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proteins, and DNA (reviewed in Refs. 21–23). The fact that memory mechanisms might be directly compromised by elevated ROS strongly supports the connection between AD and oxidative stress and underscores the importance of establishing how ROS may be coupled to other major aspects of AD pathology. Previous investigations have focused on stimulation of ROS production within neurons by fibrillar Aβ, the major component of senile plaques. Although insoluble fibrils are hallmarks of AD pathology, a newer aspect receiving considerable attention is the build-up of soluble Aβ oligomers in the brains and cerebrospinal fluid of AD patients (24–29). These oligomers (also known as ADDLs) are gain-of-function pathogenic ligands that attack postsynaptic spines, block LTP, and impact molecular mechanisms related to synaptic plasticity and memory formation (24–26, 29), and their accumulation in the brains of affected individuals has been suggested to explain the early cognitive decline characteristic of AD (30). However, despite increasing evidence indicating that soluble Aβ oligomers are the proximal neurotoxins in AD, a direct causal link between ADDLs and neuronal oxidative stress has not yet been established.

We now report that ADDLs stimulate a prominent increase in ROS formation in mature hippocampal neurons in culture, thus establishing a link with a major facet of AD neuropathology. The mechanism of ADDL-stimulated ROS formation requires ADDL targeting and activation of NMDA-Rs, leading to a rapid increase in neuronal calcium levels. The NMDA-R open channel blocker memantine has been approved as a therapeutic drug for AD, because it modestly preserves memory in patients, somewhat paradoxically given that memory formation requires NMDA-R activity (31–33). Significantly, we show here that memantine blocks ADDL-induced increase in calcium levels and oxidative stress, thus providing a pathologically specific mechanism for the therapeutic value of memantine. Since dysregulation of ROS levels by ADDLs may contribute to the early memory impairment in AD, new drugs optimized as antagonists of ADDL activity would probably provide improved AD therapeutics.

**EXPERIMENTAL PROCEDURES**

**Materials**—Aβ-(1–42) was purchased from California Peptide (Napa, CA), and Aβ-(1–40) was from rPeptide (Bogart, GA). Monoclonal antibody 6E10 was from Signet Laboratories (Dedham, MA). Anti-NR1 C-terminal antibody was from Chemicon International (Temecula, CA), and anti-NR1 N-terminal antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anhydrous Me₂SO was from Sigma. Coomassie Plus protein assay and SuperSignal West Femto Maximum Sensitivity substrate were from Pierce. Cyclophilin B antibody was from Affinity Bioreagents (Golden, CO). Memantine was from Sigma.

**Preparation and Characterization of ADDLs**—Aβ-(1–42) was prepared in aliquots as a dried hexafluoro-2-propanol film and stored at −80 °C as previously described (24, 25). The peptide film was dissolved in undiluted, sterile Me₂SO to make a 5 mM solution. The solution was diluted to 100 μM with Ham’s F-12 medium without glutamine (BioSource International, Rockville, MD) and aged overnight at 4 °C. The preparation was centrifuged at 14,000 × g for 10 min at 4 °C to remove insoluble aggregates (protofibrils and fibrils), and the supernatants containing soluble Aβ oligomers were transferred to clean tubes and stored at 4 °C. For the experiment shown in Fig. 3C, ultrafiltration of ADDL preparations was performed using a 50 kDa cut-off Microcon YM-50 device (Millipore, Bedford, MA), according to the manufacturer’s instructions. Protein concentrations were determined using the Coomassie Plus protein assay and bovine serum albumin as a standard. Routine structural characterization of ADDL preparations was weekly performed by Western immunoblots using ADDL-selective antibodies (34–36) and by size exclusion chromatography.

For Western immunoblot analysis, samples were mixed 1:1 with Tricine sample buffer and resolved on a 10–20% gel with Tris/Tricine/SDS buffer at 120 V for 80 min at room temperature. The gel (20 pmol of Aβ/ lane) was electroblotted onto Hybond ECL nitrocellulose using 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, 0.02% SDS, pH 8.3, at 100 V for 1 h at 4 °C. The blots were blocked with 5% nonfat milk in Tris-buffered saline Tween 20 (TBS-T) (0.1% Tween 20 in 20 mM Tris-HCl, pH 7.5, 0.8% NaCl) for 1 h at room temperature. Anti-Aβ monoclonal antibody 6E10 or polyclonal anti-ADDLs antibody M69/2 were diluted (1:5,000 or 1:2,000, respectively) in 5% milk/TBS and incubated with the blots for 90 min at room temperature. Following three 10-min washes with TBS-T, the blots were incubated with horseradish peroxidase-linked anti-mouse or anti-rabbit IgGs (1:40,000 in TBS-T) overnight at 4 °C. The blots were washed three times for 10 min with TBS-T, rinsed three times with deionized H₂O, developed with SuperSignal West Femto Maximum Sensitivity substrate (1:1 dilution with water), and imaged on an Eastman Kodak Co. Image Station.

**Preparation of Aβ-(1–40) Monomer Solutions and Aβ-(1–42) Fibrils**—Aβ-(1–40) was prepared in aliquots as a dried hexafluoro-2-propanol film and stored at −80 °C. The peptide film was dissolved in undiluted, sterile Me₂SO to make a 5 mM solution. The solution was diluted to 100 μM with Ham’s F-12 medium without glutamine (BioSource International) and was immediately centrifuged at 14,000 × g for 10 min at 4 °C to remove any insoluble aggregates (protofibrils and fibrils). The supernatant containing Aβ-(1–40) monomers was transferred to a clean tube and used immediately.

For preparation of fibrils, an aliquot of the dried hexafluoro-2-propanol film of Aβ-(1–42) (see above) was dissolved in undiluted, sterile Me₂SO to make a 5 mM solution. This solution was 50-fold diluted in 10 mM HCl in PBS, immediately vortexed for 30 s, and incubated for 24 h at 37 °C.

**Neuronal Cultures**—Hippocampal neuronal cultures were prepared and maintained in neurobasal medium supplemented with B27 (Invitrogen) for 3 weeks as described previously (25). Cultures were treated for different times with vehicle, ADDLs (500 nM), or other experimental conditions, as indicated under “Results.”

**Cell Viability Assay**—Neuronal viabilities were assessed using the Live/Dead assay (Molecular Probes, Inc., Eugene, OR) following the manufacturer’s instructions. Randomly chosen fields were examined and counted in a Nikon Eclipse TE 2000-U microscope. Three different fields were examined per
well, and three cultures were used per experimental condition (~400 cells analyzed/experimental condition).

**Immunocytochemistry**—Primary cultures of E-18 hippocampal neurons were plated on poly-l-lysine-coated coverslips and grown for 20 days (20 DIV). Neurons were maintained for 1 h at 37 °C in the presence of vehicle, ADDLs, ADDLs + N-terminal anti-NR1 antibody, ADDLs + C-terminal anti-NR1 antibody, or ADDLs + anti-cyclophilin B antibody. When present, the antibodies were added to the medium 30 min before ADDLs, and a second addition was done right before ADDLs.

Cells were fixed by adding an equal volume of 3.7% formaldehyde (in PBS buffer) to the medium for 5 min followed by the removal of the entire fix medium solution and replacement with 3.7% formaldehyde for 10 min. Cells were rinsed 3 times with PBS, incubated with PBS, 10% normal goat serum overnight at 4 °C, and immunolabeled by overnight incubation at 4 °C with the ADDL-selective NU1 antibody (36) (1:1,000 dilution) in PBS, 10% normal goat serum. Neurons were then rinsed three times with PBS and incubated for 3 h at room temperature with Alexa Fluor 555 anti-mouse IgG secondary antibody (1:1,000 dilution; Molecular Probes, Inc., Eugene, OR) in PBS, 1% normal goat serum. Cells were rinsed five times with PBS, and coverslips were mounted with Prolong Gold mounting medium (Molecular Probes). Cells were visualized on a Nikon Eclipse TE 2000-U fluorescence microscope, and images were digitally acquired using MetaMorph software (Meta Image Series, Universal Imaging Corp.).

Quantitative analysis of the immunofluorescence data was carried out by histogram analysis of the fluorescence intensity at each pixel across the images using Image J (Windows version; National Institutes of Health) (37). Appropriate thresholding was employed to eliminate background signal in the images before histogram analysis. Cell bodies were digitally removed from the images so that only ADDL binding to dendritic arbors was analyzed. The results of the analysis of 30 images acquired in each experimental condition, carried out in triplicate (27 images, ~300 cells analyzed in total), were then combined to allow quantitative estimates of changes in neuronal ROS levels. At least three independent experiments were performed for each of the NMDA-R antagonists and antibodies tested.

**Measurement of ROS Formation**—ROS formation was assessed using 2 μM CM-H2DCFDA (Molecular Probes). Fluorescence probe loading. After that, neurons were rinsed three times with PBS and then incubated for 10 min at 4 °C, centrifuged, and washed as above. Cells were immediately visualized on the Nikon microscope in neurobasal medium without phenol red. Analysis of DCF fluorescence data was carried out using Image J (37). Appropriate thresholding was employed to eliminate background signal in the images before histogram analysis. Results from the analysis of nine images acquired in each experimental condition, carried out in triplicate (27 images, ~300 cells analyzed in total), were then combined to allow quantitative estimates of changes in neuronal ROS levels. At least three independent experiments were performed for each of the NMDA-R antagonists and antibodies tested. In all experiments, fluorescence levels were normalized by the number of cells.

To investigate the effect of the calcium chelator BAPTA on ROS production, hippocampal neurons in culture were loaded with 100 μM BAPTA-acetoxyethyl ester (Invitrogen) in calcium-free Krebs-Ringer buffer for 30 min at 37 °C. ADDLs (500 nM) were then added for 4 h, and ROS measurements were carried out as described above.

**Effects of ADDLs on Intracellular Ca2+ Levels in Hippocampal Neurons**—Primary cultures of E-18 hippocampal neurons (20 DIV) were used. Cells were loaded with 2 μM fluo-4 (Molecular Probes) for 40 min at 37 °C in the CO2 incubator using neurobasal medium supplemented with B27. Cells were then washed three times with Hanks’ basal salt solution and left for an additional 30 min at 37 °C in phenol red-free neurobasal medium in the CO2 incubator to permit complete hydrolysis of the probe. When present, 10 μM memantine or 1 μg/ml N-terminal anti-NR1 antibody was added in the last 15 min of incubation. 300 nM ADDLs or the equivalent volume of vehicle were added to the wells directly above the microscope objective. Time lapse recordings of fluo-4 fluorescence were acquired every 1 s for 90 s. Quantitative analysis of fluo-4 fluorescence data were carried out using Image J (37). Appropriate thresholding was employed to eliminate background signal in the images before histogram analysis. Experiments were carried out using three different cultures (~120 cells analyzed/experimental condition), and data were combined to allow quantitative estimates of changes in neuronal Ca2+ levels.

**Immunoprecipitation of ADDLs from Detergent-extracted Synaptosomal Membranes**—Synaptosomes were prepared from rat forebrain as described (40). Goat anti-mouse IgG (Jackson ImmunoResearch) was linked to Dynabead M-500 subcellular (Invitrogen) according to the manufacturer’s instructions. For co-isolation of synaptic proteins and ADDLs, synaptosomes (2 mg, 0.5 mg/ml) treated with vehicle or with 300 nM ADDLs in PBS for 1 h at 4 °C were centrifuged at 6,000 × g for 10 min at 4 °C and washed four times by resuspending in PBS, incubating for 10 min, and centrifuging. The synaptosomes were resuspended to 0.5 mg/ml in PBS with 2.5 μg/ml of the ADDL-selective NU-2 monoclonal antibody (36), incubated for 1 h at 4 °C, centrifuged, and washed as above. Synaptosomes resuspended to 1 mg/ml in PBS with 0.2% (v/v) Triton X-100 were incubated on ice for 30 min; deoxycholic acid was added to a final concentration of 1% (v/v).
acid was added to 0.1% (w/v) for 30 min on ice, and the mixture was incubated with anti-mouse IgG Dynabeads (3 × 10⁷ beads) overnight at 4 °C. The Dynabeads were washed 12 times with 0.3 M NaCl, 1% Triton X-100, 0.5% deoxycholic acid in PBS and then sequentially extracted for 10 min each with 50 μl of 1% deoxycholic acid at room temperature, 1% Sarkosyl on ice (indicated as SKL-1 in Fig. 3A), and again 1% Sarkosyl on ice (indicated as SKL-2 in Fig. 3A). Finally, the Dynabeads were extracted with 50 μl of 0.1% SDS at room temperature for 30 min (indicated as SDS in Fig. 3A). Samples (10 μl), mixed with an equal volume of Laemmli sample buffer, were subjected to SDS-PAGE (41) on a 4–20% Tris-glycine gel (Bio-Rad) at 120 V for 65 min at room temperature and electroblotted onto nitrocellulose (Hybond ECL; Amersham Biosciences) with 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, 0.02% SDS, pH 8.3, at 100 V for 1 h at 4 °C. Following blocking in 5% nonfat dry milk in TBS-T (20 mM Tris-HCl, pH 7.5, 0.8% NaCl, 0.1% Tween 20), the blots were probed with polyclonal antibody to ADDLs (M69-2; 1:2000) (34) or NMDA-R1 (anti-NR-1; 1:500; Santa Cruz Biotechnology) in milk/TBS-T overnight at 4 °C. The blots were washed three times for 10 min each with TBS-T and incubated with horseradish peroxidase-linked anti-rabbit IgG (1:40,000 in TBS-T; Amersham Biosciences) for 1 h at room temperature, washed three times for 10 min each with TBS-T, and visualized using SuperSignal West Femto maximum sensitivity substrates (Pierce; 1:1 dilution with water) on a Kodak Image Station. The letters V and A in Fig. 3A indicate vehicle- and ADDL-treated synaptosomes, respectively.

Dot Blot Analysis of Synaptosomal Binding of Aβ-derived Preparations—Synaptosomes were treated with 300 nM Aβ-(1–40) monomers, prepared as described above) for 1 h at 37 °C, washed, and labeled with monoclonal 6E10 antibody as described above for immunoprecipitation. The washed synaptosomes were resuspended in PBS with 0.2% Triton X-100 and incubated on ice for 30 min. Samples were diluted in PBS, and 1-μl aliquots (containing ~0.5, 0.25, or 0.125 μg of total synaptosomal protein) were spotted in duplicate on nitrocellulose and allowed to dry for 10 min. The blot was blocked with 5% nonfat dry milk in TBS-T for 45 min and then incubated with horseradish peroxidase-linked anti-mouse IgG (1:20,000 in milk/TBS-T; Amersham Biosciences) for 1 h. The blot was washed and visualized as described above for Western blots. Relative intensities were quantified using the Kodak Image Station.

RESULTS

ADDLs Induce Formation of Reactive Oxygen Species in Mature Hippocampal Neurons—Oxidative stress in neuronal cultures was evaluated using CM-H₂DCFDA, a fluorescent probe that is sensitive to the formation of various types of ROS. Our initial experiments examined the effects of Aβ oligomers on ROS formation in highly differentiated, mature (3-week-old) hippocampal neuronal cultures. Neurons treated with ADDLs showed a prominent increase in DCF fluorescence, whereas vehicle-treated neurons exhibited basal fluorescence levels corresponding to the physiological endogenous production of ROS (Fig. 1). The time dependence of ADDL-induced ROS formation detected by DCF fluorescence was also investigated; exposure to ADDLs for up to 1 h led to weak generation of ROS (not shown), whereas robust ROS responses were induced by exposure of hippocampal neurons to ADDLs for 3 or 4 h. Interestingly, a punctate DCF fluorescence pattern was observed in the dendritic arbors of ADDL-treated neurons (Fig. 1A; supplemental Fig. S1), resembling the pattern of punctate ADDL binding described previously (25, 26) and suggesting that increased ROS production might take place in the vicinity of synaptic ADDL binding sites. Stimulation of ROS generation by ADDLs was a very reproducible and robust effect and was observed in more than 30 independent experiments using different neuronal cultures and ADDL preparations. The addition of 500 μM 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, abolished the ADDL-induced ROS response, suggesting an important role of mitochondrial ROS formation in the neuronal oxidative stress (supplemental Fig. S2). Control measurements showed that neuronal viabilities were not impaired during the 4-h exposure to 2,4-dinitrophenol under our experimental conditions (supplemental Fig. S2).
**ADDL-induced Oxidative Stress Mediated by NMDA-R**

**Figure 2.** ADDL-induced ROS generation is blocked by anti-ADDLs and anti-NMDA-R antibodies. Primary cultures of E-18 hippocampal neurons (20 DIV) were maintained for 4 h at 37°C in the presence of different additions, as indicated. A–E, representative images from DCF fluorescence in cultures treated with vehicle (A), 500 nM ADDLs (B), 500 nM NU1 antibody + 500 nM ADDLs (C), 1 µg/ml N-terminal α-NR1 antibody + 500 nM ADDLs (D), or 1 µg/ml C-terminal α-NR1 antibody + 500 nM ADDLs (E). When present, antibodies were added 30 min before the addition of ADDLs. In the case of α-NR1 antibodies, a second shot of antibody was added right before the addition of ADDLs. Cells were loaded with CMH₂DCFDA as described under “Experimental Procedures.” In order to allow direct comparison of ROS levels, identical conditions and exposure times for image acquisition were employed for all experimental conditions. Scale bar, 30 µm. F, analysis of DCF fluorescence obtained from at least six independent experiments (∼100 cells analyzed/experimental condition in each experiment) using NIH Image J software (Windows version). *, indicates statistically significant (p < 0.007) differences relative to ADDL-treated cultures.

**ADDLs are ligands for postsynaptic spines, co-localizing with PSD-95 (postsynaptic density 95 kDa) (26).** Because the overactivation of PSD-95-associated NMDA receptors could instigate neuronal ROS formation, we next investigated the involvement of NMDA-Rs in ADDL-induced ROS generation. Interestingly, an antibody specific for the extracellular (N-terminal) domain of the NR1 subunit of NMDA-Rs completely blocked ROS formation induced by ADDLs (Fig. 2D), whereas a control antibody against the C-terminal (intracellular) domain of NR1 had no effect (Fig. 2E).

**ADDL Binding to Neurons Is Mediated by a Protein Receptor Complex That Includes NMDA Receptors—**Because the results presented above implicated NMDA-Rs in ADDL-induced oxidative stress, we sought to determine whether NMDA-Rs were part of the receptor complex involved in synaptic ADDL binding. To this end, we carried out immunoprecipitation assays using ADDL-treated, detergent-extracted synaptosomal membranes (see “Experimental Procedures”). These experiments showed that the NR1 subunit of NMDA-Rs co-immunoprecipitated and was released together with ADDLs during sequential detergent washes of the synaptosomal isolates (Fig. 3A).

Similar Western blot profiles were obtained for ADDLs that were sequentially extracted from ADDL-treated synaptosomes (Fig. 3A) and the initial ADDL preparations used in this study (Fig. 3B). Fig. 3B also shows a comparison of the immunoreactivities of our typical ADDL preparations with M69/2, a polyclonal anti-ADDLs antibody, and 6E10, a generic anti-Aβ monoclonal. Whereas 6E10 recognizes Aβ monomers, trimers, and tetramers and poorly reacts with higher order oligomers, M69/2 reacts strongly with higher order oligomers and also with trimers and tetramers but not with monomers.

To further investigate the specificity of the high affinity interaction between ADDLs and synaptosomes, we examined the binding of different Aβ-derived preparations to rat brain synaptosomes. To this end, we used our regular ADDL preparation, freshly prepared Aβ-(1–40) monomers, and Aβ-(1–42) fibrils. In addition, it has previously been shown that synaptic ligands found in ADDL preparations pass through 100 kDa but not through 10 kDa filters (26). Here, we have further fractionated the ADDL preparation by ultrafiltration through a 50 kDa cut-off filter, generating a filtrate fraction containing <50-kDa species and a retentate fraction containing higher order oligomers (mass > 50 kDa). After incubation of synaptosomes with these different preparations, they were extensively washed
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Results showed that the whole ADDL preparation, the \( \geq 50 \)-kDa retentate, and fibrils bind to synaptosomes, whereas \( \beta \)-monomers \((\beta-(1-40))\) and the lower molecular weight oligomers present in the \( \leq 50 \)-kDa filtrate do not bind. This indicates that pathologically relevant oligomers bind with high affinity to synaptosomal membranes, whereas low-\( N \) oligomers and physiological \( \beta \)-monomers do not.

Furthermore, to rule out the possibility that \( \beta \)-monomers or low molecular weight oligomers might be bound to synaptosomes but occluded from interaction with 6E10, we also carried out SDS-PAGE separation of the samples, followed by Western blotting and immunodetection of the heat-treated blot (100 °C, 5 min) with 6E10 (data not shown). These results verified the presence of intense \( \beta \)-immunoreactivity in ADDL-, retentate-, and fibril-treated synaptosomes but not in \( \beta \)-monomer- or filtrate-treated synaptosome samples.

Additional demonstration of the different patterns of interaction of distinct \( \beta \)-derived preparations with neurons was provided by 6E10 immunofluorescence labeling of hippocampal neurons in culture (Fig. 3C). Although ADDLs bound to neurons in a typical synaptic hot spot pattern, no binding was detected using \( \beta-(1-40) \)-monomers. These results further substantiate the notion that pathological \( \beta \)-oligomers specifically target synapses in neurons, whereas no binding of physiological \( \beta \)-monomers to neurons could be detected under our conditions. By comparison, \( \beta \)-fibrils appeared as large aggregates that do not exhibit specific synaptic binding.

Significantly, the N-terminal anti-NR1 antibody caused \( \sim 60\% \) reduction in total ADDL binding to hippocampal neurons (Fig. 3, G and H), whereas C-terminal anti-NR1 and anti-cyclophilin B antibodies, used as controls, had no effect on ADDL binding (Fig. 3, H and I). These results indicate that a large fraction of the ADDLs bind to or in close proximity to NMDA-Rs. Together with the ability of NR1 antibodies to inhibit ADDL-stimulated ROS generation, they support the

to remove unbound \( \beta \) and then labeled with 6E10 antibody. The samples were then spotted onto nitrocellulose membranes. Detection of 6E10 immunoreactivity in the dot blots served as a marker for the presence of \( \beta \) in the samples (Fig. 3C). These
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FIGURE 4. Anti-NR1 N-terminal antibody does not block NMDA-induced ROS generation. Primary cultures of E-18 hippocampal neurons (14 DIV) were maintained for 4 h at 37 °C in the presence of different additions, as indicated. A–C, representative images from DCF fluorescence in cultures treated with vehicle (A), 80 μM NMDA (B), or 1 μg/ml N-terminal α-NR1 antibody + 80 μM NMDA (C). When present, antibody was added 30 min before the addition of NMDA, and a second shot of antibody was added right before the addition of NMDA. A montage of nine fields is shown for each condition. Cells were loaded with CMH2DCFDA as described under “Experimental Procedures.” In order to allow direct comparison of ROS levels, identical conditions and exposure times for image acquisition were employed for all experimental conditions. Scale bar, 60 μm. D, analysis of DCF fluorescence obtained from four independent experiments (~100 cells analyzed/experimental condition in each experiment) using Image J software (Windows version; National Institutes of Health). *, statistically significant (p < 0.005) differences relative to control, vehicle-treated cultures.

FIGURE 5. Dose dependence of ADDL- and NMDA-induced ROS formation. A, integrated DCF fluorescence in primary cultures of E-18 hippocampal neurons (20 DIV) maintained for 3 h at 37 °C in the presence of different concentrations of NMDA, as indicated. B, integrated DCF fluorescence in primary cultures of E-18 hippocampal neurons (20 DIV) maintained for 3 h at 37 °C in the presence of different concentrations of NMDA, 500 nM ADDLs, or 500 nM ADDLs + 200 μM NMDA, as indicated. Cells were loaded with CMH2DCFDA as described under “Experimental Procedures.” In order to allow direct comparison of ROS levels, identical conditions and exposure times for image acquisition were employed for all experimental conditions. The graph represents the analysis of DCF fluorescence obtained using three different cultures (~100 cells analyzed/experimental condition) using Image J.; *, statistically significant (p < 0.01) differences.

Conclusion that ADDL binding to specific sites at neuronal processes is required for stimulation of ROS generation.

NMDA-induced ROS Generation Is Not Blocked by Anti-NR1 Antibody—Additional measurements showed that exposure of neuronal cultures to 80 μM NMDA induced ROS formation similar to that induced by exposure to 500 nM ADDLs (Fig. 4, A, B, and D). Importantly, ROS formation induced by NMDA was not affected by the addition of the N-terminal anti-NR1 antibody (Fig. 4, C and D), ruling out the possibility that the blockade of ADDL-induced ROS formation might be due to impairment of receptor function in the presence of the antibody. This result also implies that the stimulation of ROS formation induced by ADDLs is not indirectly caused by an increase in glutamate release from damaged neurons in culture.

Dose Dependence of ADDL- and NMDA-induced ROS Formation—We next investigated the dose dependences of ROS generation induced by ADDLs or NMDA. Increasing the concentration of ADDLs from 100 nM to 1 μM resulted in a dose-dependent increase in ROS (Fig. 5A). Higher ADDL concentrations were not utilized in our assays due to the increased possibility of protofibril and/or fibril formation when ADDLs are incubated at higher concentrations at 37 °C in culture medium. ROS formation induced by NMDA was also found to be dose-dependent (Fig. 5B). When ADDLs and NMDA were added simultaneously to the cells, we found a small, nonadditive increase in ROS formation compared with NMDA alone (Fig. 5B), consistent with the notion that ADDL-induced ROS formation largely occurs via activation of NMDA receptors.

Memantine Protects against ADDL-induced Oxidative Stress—Memantine is an NMDA-R open channel blocker recently approved for AD treatment. However, the relationship of its therapeutic mechanism to Alzheimer’s pathology is incompletely understood. In particular, it is not sufficiently clear why a drug that blocks NMDA-Rs, which play essential roles in synaptic plasticity and LTP, might be beneficial in terms of preserving memory in AD patients. In order to gain further insight into the mechanisms of action of memantine, we next investigated the hypothesis that this drug might protect against neuronal damage initiated by soluble Aβ oligomers. At doses comparable with its clinical pharmacological window, memantine potently inhibited ADDL-induced ROS formation in hippocampal neurons (Fig. 6, A–D), suggesting that one possible mechanism of action of memantine involves prevention of neuronal oxidative stress instigated by ADDLs. It is interesting to note, however, that memantine did not block ADDL binding to hippocampal neurons (Fig. 6, E–H), suggesting that ADDLs do not bind directly to the NMDA-R channel pore.

Other NMDA-R Antagonists Also Block ADDL-induced ROS Formation—To further investigate the mechanisms by which ADDLs lead to NMDA-R dysfunction and excessive ROS gen-
eration, we investigated the effects of another channel blocker, MK-801, and of a competitive antagonist, APV, on ROS formation in hippocampal neurons. Similar to memantine, both compounds completely blocked ADDL-induced ROS formation (Fig. 7, A–D). Interestingly, however, APV (but not MK-801) significantly reduced ADDL binding to hippocampal neurons (Fig. 7, E–H). Inhibition of ADDL binding by APV is consistent with the inhibition of ADDL binding by an anti-NR1 antibody (described above). The agonist, NMDA, had no effect on ADDL binding (Fig. 7H). These results suggest that the APV coordination domain within the agonist binding site may either represent a direct ADDL binding domain or be involved, via the induction of protein conformational changes, in the regulation of ADDL interactions with the NMDA-R or with closely associated protein receptors.

**DNQX Does Not Block ADDL-induced ROS Formation**—We also examined the effect of the AMPA receptor antagonist,
DNQX, on ADDL-induced ROS formation. Treatment of hippocampal cultures with 10 μM DNQX caused only a slight inhibition of ROS formation (Fig. 7D) and did not block ADDL binding to neurons (Fig. 7H), excluding a direct participation of AMPA receptors in ADDL-induced oxidative stress. This result also corroborates the notion that ADDL-induced ROS formation is not indirectly caused by excessive release of glutamate to the medium by damaged neurons.

**ADDLs Induce an Increase in Intracellular Ca^{2+} Levels, Which Is Blocked by Memantine and Anti-NR1**—We next investigated the status of NMDA receptors with respect to their functional ability to mobilize calcium in ADDL-treated neurons. We found that 300 nM ADDLs induced a rapid, transient increase (~3.5-fold) in calcium levels in hippocampal neurons, compared with vehicle-treated cultures (Fig. 8). Both memantine and anti-NR1 antibody blocked this effect (Fig. 8C), implicating the NMDA-R in the rapid ADDL-induced calcium increase.

**Intracellular Calcium Chelation Blocks ADDL-induced ROS Formation**—In order to investigate the connection between ADDL-induced elevation in intraneuronal calcium levels and ROS formation, experiments were performed in which ADDLs were added to hippocampal neurons that had been previously loaded with the cell-permeant calcium chelator, BAPTA-acetoxymethyl ester. The increase in ROS levels instigated by ADDLs was totally blocked in BAPTA-loaded neurons (Fig. 8D), indicating that NMDA-mediated calcium influx gives rise to ROS formation in ADDL-treated neurons.

Although BAPTA has been extensively used as a calcium chelator, it is known that BAPTA and BAPTA-derived indicators (including Fluo-4) also bind zinc and other heavy metals (42). In our experimental conditions, however, we found that the measured increases in Fluo-4 signal could be specifically blocked by memantine and anti-NR1 antibodies, suggesting that the signal indeed originates from NMDA-mediated calcium influx.

**DISCUSSION**

Under physiological cellular conditions, ROS are produced at low levels largely due to mono-electronic reduction of oxygen (generating superoxide) at the mitochondrial respiratory chain (1, 2). It has been demonstrated that low levels of ROS are necessary components of signal transduction cascades in a number of normal cellular processes (18, 43). Of particular interest, ROS have been implicated in memory-related mechanisms, as shown by their requirement for LTP (18, 43). Therefore, even relatively subtle changes in the regulation of ROS levels may have important deleterious consequences. Moreover, high levels of ROS, generated when their rate of production exceeds cellular scavenging capacity, have been implicated as damaging toxic molecules in the age-related impairments of LTP (18, 43).

There is a clear involvement of oxidative stress in AD (44). In vitro studies indicate that cell exposure to aggregated Aβ leads to toxicity due to calcium influx and the induction of oxidative free radical damage (20, 45). Several different hypotheses have been proposed to explain this toxic effect, including the formation of ion channels in cell membranes (46), the spontaneous fragmentation of Aβ to generate peptidyl radicals (47), and the direct formation of H₂O₂ by Aβ (45). In addition, it has been proposed that the mechanism of Aβ-induced oxidative stress and neurotoxicity involves methionine-associated formation of ROS (48). One current controversy in the amyloid hypothesis is whether or not amyloid fibrils are required for toxicity. Interestingly, oxidative stress significantly precedes the appearance of amyloid plaques and neurofibrillary tangles in AD brains (reviewed in Ref. 49) and also precedes fibrillar deposition of Aβ in a transgenic Caenorhabditis elegans model (50). These observations are consistent with the concept that the toxic species involved in ROS formation are prefibrillar.

In the present study, we have established that ADDLs instigate neuronal ROS formation well above the physiological levels and that this can be inhibited by an ADDL-selective monoclonal antibody that prevents ADDL binding to neurons. This result gives support to the notion that oligomerization of Aβ leads to a pathogenic gain-of-function associated with various forms of neuronal damage (30), including oxidative stress. It is...
interesting to note that ADDL binding to neurons occurs in a punctate fashion along the dendrites, at sites previously identified as PSD-95-enriched synapses (26). This is similar to the punctate ROS fluorescence labeling that can be observed in high magnification images of ADDL-treated neurons (Fig. 1A and supplemental Fig. S1), suggesting that excessive ROS are locally generated in response to synaptic ADDL binding. The finding that ROS formation can be totally blocked by the mitochondrial uncoupler, 2,4-dinitrophenol (supplemental Fig. S2) suggests that mitochondria play an important role in ADDL-induced oxidative stress. In this regard, mitochondria are known to accumulate in synapses (51, 52), and a very recent study has shown that mitochondrial trafficking to synapses is dynamic and regulated by synaptic activity (53). Of considerable interest, that study also showed that mitochondria in dendritic synapses undergo marked morphological remodeling in neurons subjected to a neurotoxic glutamate insult (53). Taken together, these observations suggest that dysregulation of NMDA-R function induced by ADDL binding to neuronal synapses may lead to synaptic mitochondrial dysfunction and excessive ROS formation.

An antibody against the extracellular N-terminal domain of the NR1 subunit of NMDA-Rs completely blocked ADDL-stimulated ROS formation (Fig. 2, D and F), whereas a C-terminal (intracellular) antibody used as a control was without effect (Fig. 2, E and F). It is noteworthy that binding of the N-terminal antibody to the NR1 subunit of NMDA-Rs did not cause functional impairment of the receptor, as indicated by the fact that ROS formation induced by NMDA was unaffected by the antibody (Fig. 4). Importantly, the N-terminal anti-NR1 antibody reduced ADDL binding by ~60% (Fig. 3, G and I). We have previously shown that more than 90% of ADDL immunoreactivity in mature hippocampal cultures coincides with PSD-95 (25), a scaffolding protein that tethers NMDA receptors to postsynaptic densities. These results suggest that ADDLs bind specifically to synaptic spines, at or in the close vicinity of NMDA-Rs, and trigger excessive ROS formation. In accord with this hypothesis, we found that the NR1 subunit of NMDA receptors co-immunoprecipitates with ADDLs from detergent-extracted ADDL-treated rat synaptosomal membrane preparations (Fig. 3A). Thus, although there may be other types of ADDL receptors in neurons (as suggested by the fact that the anti-NR1 antibody does not completely abolish ADDL binding), these observations indicate that NMDA-Rs play a pivotal role in ADDL-instigated neuronal damage.

Results presented here also indicate that ADDLs (and, in particular, the ≥50-kDa oligomer species present in ADDL preparations) specifically bind with high affinity to synaptosomal membranes, whereas Aβ-(1–40) monomers and low molecular mass (∼50-kDa) oligomers do not. This finding suggests that development of therapeutics to prevent ADDL-instigated neuronal oxidative stress would benefit from targeting higher molecular weight oligomers and their interaction with synapses.

Memantine is an open channel, uncompetitive NMDA-R inhibitor recently approved for treatment of AD. Memantine modestly preserves memory in patients with moderate to severe forms of AD (54–56), and its beneficial actions have so far been related to the prevention of excessive NMDA-R activation (i.e. excitotoxicity) (57). According to that view, the efficacy of memantine in neurological diseases associated with NMDA-R overactivation is related to prevention of the excessive influx of Ca^{2+} through the receptor’s associated ion channel, leading to ROS formation (57). However, the relationship of the therapeutic mechanism of action of memantine to Alzheimer disease pathology is still incompletely understood (18, 32), since NMDA-R function is known to be essential for spatial learning and memory and for the induction of long term synaptic plasticity (31–33) (reviewed in Ref. 58). We now show that memantine protects against neuronal oxidative damage initiated by ADDLs (Fig. 6). In this regard, an important issue we investigated here is whether neuronal oxidative stress is directly instigated by activation of NMDA-Rs by ADDLs or might be indirectly caused by overactivation of glutamate receptors by excessive glutamate released from ADDL-damaged neurons. Glutamate-induced excitotoxicity involves over-activation of NMDA-Rs, which in turn requires coincident activation of AMPA/kainate receptors by glutamate. Our finding that DNQX, an AMPA receptor antagonist, causes only a slight reduction in ADDL-induced ROS formation (Fig. 7) suggests that AMPA receptors are not importantly involved in this process, favoring the hypothesis of direct activation of NMDA-Rs by ADDLs. This conclusion is further supported by the finding that the anti-NMDA-R antibody blocked ADDL-induced but not NMDA-induced ROS formation (Figs. 2 and 4), indicating that blockade was not caused by functional inhibition of the receptor but rather by prevention of its activation by ADDLs.

The finding that the anti-NR1 antibody blocks ADDL-induced toxicity mediated by NMDA receptors without blocking normal NMDA receptor function may have important implications for drug development in AD. As noted above, AD is currently treated by an NMDA receptor antagonist. Our present findings establish proof of concept for developing a superior therapeutic that protects NMDA receptor function while blocking against ADDL toxicity.

We also found that ADDL- and NMDA-induced ROS formation are dose-dependent and nonadditive, suggesting that ADDL-induced ROS formation largely occurs via overactivation of NMDA receptors. However, this does not rule out the possibility that another, quantitatively less important mechanism(s) can also contribute to ROS generation instigated by ADDLs.

Treatment with ADDLs induced a rapid and transient increase in intracellular calcium levels in mature hippocampal neurons (Fig. 8). In line with a recent report (59), the increase in Ca^{2+} levels observed in the presence of Aβ oligomer preparations was a very rapid process, triggered a few seconds after ADDLs were added to the cells. Aβ oligomers have also been shown to be responsible for calcium influx, calpain activation, and dynamin 1 degradation mediated by NMDA receptors (60). We show here that both memantine and anti-NR1 antibody blocked the elevation in Ca^{2+} levels induced by ADDLs (Fig. 8). Moreover, we show that ADDL-induced ROS formation is totally blocked in BAPTA-loaded hippocampal neurons (Fig. 8D). These results provide a mechanistic link between activa-
tation of NMDA-Rs, calcium influx, and ROS formation induced by ADDLs.

Although NMDA-Rs are thought to be memantine targets in AD brain, memantine is also an inhibitor of a7 nicotinic acetylcholine receptors (61), which have been implicated in the down-regulation of NMDA-Rs induced by Aβ (62). However, a7 nicotinic receptors are most commonly observed on GABAergic neurons in hippocampal cultures (63), and very recent results from our group indicate that ADDLs do not bind to GABAergic cells (38). These considerations suggest that the beneficial effects of memantine we now report derive from inhibition of NMDA-Rs rather than nicotinic receptors.

Prevention of excessive NMDA-R activity is considered to be one potential route for treatment of memory loss associated with AD (55). Our current results indicate that excessive NMDA-R activity is a consequence of the neuronal impact of ADDLs and suggest that memantine helps AD patients by protecting synapses against ADDL-induced neuronal oxidative stress. Because of the important roles of low levels of ROS in physiological mechanisms related to synaptic plasticity, we propose that dysregulation of NMDA-R activity and oxidative stress may have a dual deleterious role in AD. In an early phase, oxidative stress impairs memory by interference with molecular mechanisms of plasticity, whereas in later stages of the disease, oxidative stress is mainly related to neuronal degeneration and death. These findings strongly suggest that discovering better means to inhibit the neuronal impact of ADDLs might prove a successful strategy for improved AD therapeutics.

REFERENCES