The Effects of Amyloid Precursor Protein on Postsynaptic Composition and Activity

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Hyang-Sook Hoe, Zhanyan Fu, Alexandra Makarova, Ji-Yun Lee, Congyi Lu, Li Feng, Aheadeh Pajoohesh-Ganjii, Yasuji Matsuoka, Bradley T. Hyman, Michael D. Ehlers, Stefano Vicini, Daniel T. S. Pak, and G. William Rebeck

From the Departments of Neuroscience, Physiology and Biophysics, Pharmacology, and Neurology, Georgetown University Medical Center, Washington, D. C. 20057-1464, Harvard Medical School, Massachusetts General Hospital, Massachusetts Institute for Neurodegenerative Disorders, Charlestown, Massachusetts 02129, and Howard Hughes Medical Institute, Department of Neurobiology, Duke University Medical Center, Durham, North Carolina 27710

The amyloid precursor protein (APP) is cleaved to produce the Alzheimer disease-associated peptide Aβ, but the normal functions of uncleaved APP in the brain are unknown. We found that APP was present in the postsynaptic density of central excitatory synapses and coimmunoprecipitated with N-methyl-D-aspartate receptors (NMDARs). The presence of APP in the postsynaptic density was supported by the observation that NMDARs regulated trafficking and processing of APP; overexpression of the NRII subunit increased surface levels of APP, whereas activation of NMDARs decreased surface APP and promoted production of Aβ. We transfected APP or APP RNA interference into primary neurons and used electrophysiological techniques to explore the effects of APP on postsynaptic function. Reduction of APP decreased (and overexpression of APP increased) NMDAR whole cell current density and peak amplitude of spontaneous miniature excitatory postsynaptic currents. The increase in NMDAR current by APP was due to specific recruitment of additional NR2B-containing receptors. Consistent with these findings, immunohistochemical experiments demonstrated that APP increased the surface levels and decreased internalization of NR2B subunits. These results demonstrate a novel physiological role of postsynaptic APP in enhancing NMDAR function.

Alzheimer disease (AD) is an age-related neurodegenerative disorder characterized by the progressive loss of synapses and neurons and by the formation of amyloid plaques and neurofibrillary tangles. Amyloid plaques are composed predominantly of the Aβ peptide, a 40- or 42-amino acid cleavage product of amyloid precursor protein (APP). APP is a transmembrane protein of unknown function that undergoes extracellular cleavage by one of two activities, α- or β-secretase, resulting in the formation of large N-terminal extracellular fragments of secreted APP and smaller, membrane-bound C-terminal fragments. If the initial cleavage event occurs via β-secretase, then subsequent cleavage of the C-terminal fragment by γ-secretase results in the production of Aβ.

Clues to APP function may be gleaned from studies of its different cleavage products or isoforms. Soluble forms of APP have been found to be neurotrophic, and some splice variants have protease inhibitor activity. The APP intracellular domain may alter gene transcription in conjunction with cytoplasmic proteins. In addition, the Aβ peptide has been shown to inhibit glutamate receptor activity. However, the function of full-length APP is undefined, and it is likely that full-length APP performs distinct roles from any its cleavage products. One recent study showed that mice lacking APP have impaired development of neuromuscular junctions, suggesting an important role for APP in maintaining active synapses. Understanding APP function in central neurons may provide valuable information in generating interventions against the generation of Aβ and AD pathogenesis and its accompanying memory loss.

Further evidence for a synaptic function of APP comes from studies indicating that APP processing is regulated by synaptic activity. Neuronal activity increased production of Aβ in hippocampal slices and primary neurons. This effect on APP processing may be due to activation of the NMDA subtype of glutamate receptors, which inhibited α-secretase cleavage of APP in primary neurons and in the brain and would therefore favor β-secretase cleavage and Aβ production. This mechanism of action is still controversial, since another study showed that NMDAR activation led to increased α-cleavage of APP.

NMDARs are critical for many forms of synaptic plasticity, including hippocampal CA1 long term potentiation and long-term depression.

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term depression, and consist of an obligate NR1 subunit and variable NR2 or NR3 subunits (11, 12). The NR2 subunit type determines important pharmacological and biophysical properties of NMDARs (13–16). NMDARs may also play an important role in AD, since memantine, an NMDAR antagonist, is a drug approved for moderate to serious forms of this disease.

We now report a novel physical and functional interaction between NMDARs and APP. In contrast to the inhibitory effect of Aβ on glutamatergic function, full-length APP promoted NMDAR activity in primary hippocampal neurons, as measured by increased whole cell current density, surface expression of NMDAR, and analysis of spontaneous miniature excitatory postsynaptic current (mEPSC) through NMDAR. Consistent with this role, reduction of endogenous APP levels via RNA interference (RNAi) decreased NMDAR current density and surface levels. Conversely, NMDAR expression and activity levels regulated APP trafficking and processing in primary neurons. These data reveal synaptic functions of APP and demonstrate the reciprocal effects of NMDARs and APP on protein trafficking and metabolism.

MATERIALS AND METHODS

Vector Construction—N-terminal tagged GFP for NR1, NR2A, and NR2B was used for cell surface and cell internalization assays (17). We produced full-length APP770 with a N-terminal GFP tag or a C-terminal of Myc tag. We also produced an M761V construct of GFP-tagged full-length APP770 using site-directed mutagenesis (Stratagene) and a C-terminal Myc-tagged construct of APP C99. To generate the APP short interfering RNA constructs, we used pSuper vector, which expresses an siRNA under the control of the H1 promoter and can be used for prolonged suppression of specific gene expression (18). Using Invitrogen BLOCK-iT™ RNAi Designer to identify sequences within the cDNA for silencing, APP-siRNA sequences were targeted against rat APP open reading frame (NM_019288). The sequences for the RNAi used in this study were as follows: number 7, GGTGCCATGTTGGTGAGTT; number 14, GCACTAAACTTGCACGACTA; number 37, GCCGTGTACATCCAAAGTTT.

Animals—1-month-old wild-type C57BL/6J and APP knockout mice B6.129S7-APP<sup>tm1DBo</sup>/J were purchased from Jackson Laboratory. 1-month-old Sprague-Dawley rats were purchased from Taconic. All animal protocols were approved by the Georgetown University Institutional Animal Use and Care Committee and were in compliance with the standards stated in Ref. 48.

Synaptosomal Preparations—Synaptosome fractionation was performed as described previously (19) with minor modifications. Briefly, brains from 3-month-old, wild-type C57BL/6J and APP knock-out B6.129S7-APP<sup>tm1DBo</sup>/J mice were homogenized in 0.32 m sucrose, 4 m Hepes-NaOH, pH 7.3, with protease inhibitors and centrifuged at 1000 × g for 10 min to recover the supernatant S1 and the pellet P1. S1 fraction was centrifuged at 12,000 × g for 15 min to obtain the pellet P2 (crude synaptosome) and the supernatant S2. P2 fraction was treated with an osmotic shock by diluting with double-distilled water and further centrifuged at 25,000 × g for 20 min to generate the pellet LP1 and the supernatant LS1. The LS1 fraction was subjected to ultracentrifugation to obtain the synaptic vesicle fraction. LP1 was detergent extracted in buffer B (0.16 m sucrose, 5 mm Tris-HCl, pH 8.0, 0.5% Triton X-100, 0.5 mm β-ME, 1 mm EDTA, and protease inhibitors) and then centrifuged at 33,000 × g for 20 min. The pellet LP1P was resuspended and applied to a discontinuous sucrose gradient consisting of 1.0, 1.5, and 2.0 m sucrose layers. After ultracentrifugation (200,000 × g for 2 h), the postsynaptic density (PSD) fraction was recovered at the interface between 1.5 and 2.0 m sucrose.

Cell Lines and Culture Conditions—COS7 cells were maintained in Opti-MEM (Invitrogen) with 10% fetal bovine serum (Invitrogen) in a 5% CO<sub>2</sub> incubator. COS7 cells were transiently transfected with 0.5–1 μg of plasmid in FuGENE6 (Roche Applied Science) according to the manufacturer’s protocol and cultured for 24 h in DMEM containing 10% fetal bovine serum. For co-transfections, cells were similarly transfected with 0.5–1 μg of each plasmid in Fugene 6 (Roche Applied Science) and cultured 24 h in DMEM with 10% fetal bovine serum.

Antibodies—The following antibodies were used: anti-GFP (Sigma), anti-GABA-A (Chemicon International, Temecula, CA), anti-synaptophysin (Sigma), anti-synapsin, and anti-PSD-95 (Chemicon International). For analysis of APP, we used 6E10 (Signet, Dedham, MA), 22C11 (Chemicon International), and rabbit anti-APP (Sigma); Dr. Paul Mathews (Nathan S. Kline Institute, Orangeburg, NY) provided antibodies c1/6.1 and M3.2; and Dr. Sam Gandy (Thomas Jefferson University, Philadelphia, PA) provided antibody 369, which all recognize the C terminus of APP. Antibodies against NR1 (monoclonal, recognizing amino acids 656–811), NR2A (polyclonal, against amino acids 934–1142), and NR2B (monoclonal, recognizing amino acids 934–1457) were generous gifts of Dr. Barry Wolfe (Georgetown University).

APP Analysis—Cell-associated human and rodent APP was measured in Western blots with the APP N-terminal antibody (Sigma), 22C11 (Chemicon), or C1/6.1. Rodent APP was also measured in Western blots with antibody M3.2. Rodent Aβ(1–40) in conditioned media was measured by ELISA, as described (20). Aβ(1–42) peptide was purchased from American Peptide (dissolved in DMSO). For immunostaining of APP, cells were fixed in cold (−20 °C) methanol for 10 min. After fixation, cells were washed with PBS and incubated with anti-APP and anti-PSD-95 antibody overnight at 4 °C. After primary incubation, cells were washed with PBS and then incubated with Alexa Fluor 488 (green color) goat anti-rabbit antibody (Molecular Probes) and Alexa Fluor 555 (red color) goat anti-mouse antibody for 1 h at room temperature. Stained cells were viewed with a confocal laser-scanning microscope.

Co-immunoprecipitation—Adult wild-type mouse brains or APP knock-out mouse brains were perfused with PBS and lysed in buffer containing 50 mm Tris-HCl (pH 8.0), 0.15 m NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, phosphatase, and protease inhibitors. For immunoprecipitation, lysates were incubated for 2 h at 4 °C with the anti-APP (22C11) antibody or an anti-NR1 antibody bound to protein G-Sepharose beads (Amersham Biosciences). The precipitates were then washed three times with buffer and resuspended in SDS sample buffer. The samples were separated by SDS-PAGE on 4–15% polyacrylamide gels, transferred electrophoretically to nitrocellulose.
membranes, and blocked with 5% nonfat dry milk. The blots were incubated with antibodies at room temperature for 1 h. Horseradish peroxidase-conjugated secondary antibodies were visualized by the ECL detection system and exposed to film.

**Primary Neuron Culture and Transfection**—Hippocampal neurons from embryonic day 18–19 Sprague-Dawley rats were cultured at 150 cells/mm² as described previously (21). Neurons were transfected at 10 or 12 days in vitro (DIV) by calcium phosphate precipitation (4–5 μg of DNA/well). 8–14 days after transfection, we analyzed cell surface expression levels of APP and NMDARs or the endocytosis of APP and NMDARs in vitro (DIV 16–21). Cerebellar granule cells (CGCs) were cultured from postnatal day 5–7 mouse pups and plated at 0.8–1.0 × 10⁶ cells/ml, as described previously (22, 23). CGCs were transfected at 5 days in vitro by calcium phosphate precipitation in a 4-well dish (3 μg of DNA/well). Whole cell patch clamp recordings were performed 2–3 days after transfection.

Primary cultures of mouse CGCs were prepared from postnatal day 5–7 mice and plated in a culture medium containing 25 mM KCl (23). At DIV 5, the medium was replaced with low (5 mM)-serine (all from Sigma) glucose, 0.25 mM tetrodotoxin in extracellular solution and NMDARs or the endocytosis of APP and NMDARs in vitro (DIV 16–21). Surface protein assay—Immunostaining of surface APP, NR1, and NR2B was performed as described previously (25). Briefly, live neuronal cultures were incubated with antibodies directed against extracellular N termini of human APP or GFP (10 μg/ml in conditioned medium) for 10 min to specifically label surface receptors and then lightly fixed for 5 min in 4% paraformaldehyde (nonpermeabilizing conditions). After fixation, the surface-remaining antibody-labeled APP or GFP was measured with Alexa Fluor 555-linked α-mouse secondary antibodies for 1 h. Immunostaining was quantified using Meta morph analysis of immunostaining intensity from z-stacked images from a Zeiss LSM510 confocal microscope (25). Surface localization of staining was also confirmed visually from these images.

Internalization Assay—Immunostaining of endocytosis of GFP-tagged NR2 subunits was performed as described (18). Live neuronal cultures were incubated with antibodies directed against extracellular N termini of human GFP or APP (10 μg/ml in conditioned medium) for 10 min to specifically label surface receptors and then lightly fixed for 5 min in 4% paraformaldehyde (nonpermeabilizing conditions). After fixation, the surface-remaining antibody-labeled APP or GFP was measured with Alexa Fluor 555-linked α-mouse secondary antibodies for 1 h. Immunostaining was quantified using Metamorph analysis of immunostaining intensity from z-stacked images from a Zeiss LSM510 confocal microscope (25). Surface localization of staining was also confirmed visually from these images.

Quantification and Image Analysis—Images were collected using a Zeiss LSM510 confocal microscope (Carl Zeiss, Thornwood, NY). Confocal z-series image stacks encompassing entire neurons were analyzed using Metamorph software (Universal Imaging Corp., Downingtown, PA). For measures of surface or internalized APP, dendrites from hippocampal neurons were carefully traced, and surface fluorescence intensities were determined for the traced region.

**Biotin-labeled Cell Surface Proteins**—Primary cortical neurons were treated with NMDA (10 μM) and MK801 (10 μM). After 24 h, cells were washed, and surface proteins were labeled with sulfo succinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate at 500 μg/ml in PBS, including 1 mM MgCl₂ and CaCl₂ (Pierce) under gentle shaking at 4 °C for 30 min. After quenching, cells were lysed, disrupted by sonication, and clarified by centrifugation (10,000 × g; 2 min). To isolate biotin-labeled proteins, lysate was added to immobilized NeutrAvidin TM Gel and incubated for 1 h at room temperature. Gels were washed and incubated with SDS-PAGE sample buffer, including 50 mM dithiothreitol. Eluants were analyzed by immunoblotting.

**Whole Cell Recordings**—Coverslips with CGCs were placed on the stage of an inverted microscope (TM2000; Nikon) equipped with fluorescent and phase-contrast optics. All recordings were performed at room temperature (24–26 °C) from neurons maintained for 7–8 days in vitro. Continuously perfused extracellular solution contained 145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM Hapes, 5 mM glucose, 0.25 mg/liter phenol red, and 10 μM D-serine (all from Sigma) adjusted to pH 7.4 with NaOH. Osmolarity was adjusted to 325 mosmol/liter with sucrose. NMDA was applied in a Ca²⁺- and Mg²⁺-free extracellular solution to prevent Ca²⁺-mediated inactivation of NMDAR channels. Electrodes were pulled in two stages on a vertical pipette puller to a resistance of 5–8 Mohm from borosilicate glass capillaries (Wiretrol II, Drummond, Broomall, PA) and filled with recording solution containing 145 mM potassium gluconate, 10 mM Hapes, 5 mM MgATP, 0.2 mM NaGTP, and 10 μM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, adjusted to pH 7.2 with KOH. Whole cell voltage clamp recordings from CGCs were made at −60 mV and performed at room temperature using an Axopatch 200 or an Axopatch-1D amplifier (Axon Instruments, Union City, CA). A transient current response to a hyperpolarizing 10-mV pulse was used to assess membrane input resistance and capacitance throughout the recordings. Currents were filtered at 2 kHz with an 8-pole low pass Bessel filter (Frequency Devices, Haverhill, MA), digitized at 5–10 kHz using an IBM-compatible microcomputer equipped with Digidata 1322 A data acquisition board and pCLAMP9.2 software (both from Axon Instruments). Off-line data analysis, curve fitting, and figure preparation were performed with Clampfit 9 software and Minianalysis software (Synaptosoft, Decatur, GA).

NMDA-mEPSCs were pharmacologically isolated using 25 μM bicuculline metabemidrome, 0.5 μM tetrodotoxin, and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) (5 μM) (all from Sigma) in a Mg²⁺-free solution. AMPA-mEPSCs were recorded in the presence of 25 μM bicuculline metabemidrome, 0.5 μM tetrodotoxin in extracellular solution with Mg²⁺ with a holding potential at −60 mV. The decay of NMDA-mEPSC was fitted using Clampfit 9 (Axon Instruments) from averages of several events selected with Minianalysis. The decay phase of currents was fitted using a simplex algo-
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![Figure 1](image)

**FIGURE 1.** APP is localized to synapses. A, after 14 days in culture, primary hippocampal neurons were fixed and immunostained for APP (with rabbit anti-APP antibody (Sigma)) (A1) and for PSD-95 (A2). Co-localization of APP and PSD-95 appears yellow in the right panels (A3). Dendritic segments at higher magnification are shown below each image. Scale bars, 10 μm (top) and 5 μm (bottom). B, primary hippocampal neurons from wild-type (wt) mice (top) and APP knock-out (ko) mice (bottom) were immunostained as above for APP (red) and PSD-95 (green). C, immunoblot analysis of APP (rabbit polyclonal and C1/6.1 monoclonal antibodies), NR1, synapsin, synaptophysin, and PSD-95 in brain homogenate fractions of presynaptic vesicles (SV) and PSDs from wild-type mice (left) and APP knock-out mice (right).

A double exponential fitting routine with a double exponential equation of the following form,

\[ I(t) = I_1 \exp(-t/\tau_1) + I_2 \exp(-t/\tau_2) \]  

(Eq. 1)

where \( I_x \) represents the peak current amplitude of a decay component and \( x \) is the corresponding decay time constant. To allow for easier comparison of decay times between experimental conditions, the two decay time components were combined into a weighted time constant.

\[ \tau_w = \left( I_1/(I_1 + I_2) \right) \times \tau_1 + \left( I_2/(I_1 + I_2) \right) \times \tau_2 \]  

(Eq. 2)

**Statistical Analyses**—All data are expressed as means ± S.E. Data were analyzed using analysis of variance with Graphpad Prism 4 software, using Tukey’s multiple comparison test for post hoc analyses with significance determined as \( p < 0.05 \). Electrophysiology data were analyzed by using unpaired Student’s \( t \) test (two-tailed) with Bonferroni corrections.

**RESULTS**

**APP Is Part of the Postsynaptic Density**—Immunostaining of endogenous APP in primary rat hippocampal neurons at DIV 14 demonstrated that APP was expressed at puncta along neuronal processes, which co-localized with synaptic markers PSD-95 (Fig. 1A) and synaptophysin (data not shown). As a control, we immunostained primary hippocampal neurons from wild-type and APP knock-out mice with the same antibody. As expected, APP knock-out cultures showed dramatically reduced APP immunostaining compared with controls while still showing evidence of PSD-95 puncta (Fig. 1B). Previous studies have shown that APP is presynaptic but have not determined whether it is also postsynaptic (26–28). Using synapticosomal preparations from brains of wild-type and APP knock-out mice, we generated synaptic vesicle and PSD fractions. Synaptic vesicle fractions contained synaptophysin and synapsin, and PSD fractions contained PSD-95 and the NMDAR subunit, NR1, as expected (Fig. 1C). As detected with two APP antibodies (monoclonal C1/6.1 and rabbit polyclonal anti-APP), APP was present in both the synaptic vesicle and PSD fractions (Fig. 1C, left). No APP immunoreactive bands were present in the preparations from APP knock-out mouse brain (Fig. 1C, right), demonstrating the specificity of the antibodies for APP and not for other APP family members. Isolations of pre- and postsynaptic fractions were again demonstrated by the presence of specific markers, although we did not attempt to determine whether absolute levels of these markers were altered in APP knock-out mice. These data demonstrate both a pre- and postsynaptic localization of APP.

**APP Associates with NMDARs**—As an independent approach to defining whether APP is present postsynaptically, we tested whether there was a physical association between APP and proteins of the postsynaptic density. We performed co-immunoprecipitations from whole brain lysates, using the anti-369 and a nonspecific IgG as a negative control. Immunoprecipitation of APP resulted in the co-precipitation of PSD-95 and NR1 (Fig. 2A). Neither protein was precipitated from wild type brain lysates with the IgG (Fig. 2A). As an additional negative control, immunoprecipitation with the APP antibody did not recover NR1 from lysates from brains of mice that lacked APP and the related family member APLP1 (data not shown). The nonsynaptic GABA<sub>A</sub> receptor did not co-precipitate with APP in lysates from wild-type mice (Fig. 2A), further supporting the synaptic localization of APP. We also tested whether APP associated with a postsynaptic marker in primary neurons. We found that APP co-precipitated with an antibody against NR1 but not with a control antibody (Fig. 2B). Similarly, immunoprecipitation of APP resulted in co-precipitation of NR1 (Fig. 2C, lane 2).

**NMDA Receptor Activity Regulates APP Processing**—As a third test of whether APP was present in the PSD, we examined whether manipulating postsynaptic neuronal activity affected APP trafficking and processing. Based on a previous study showing that NMDA receptor activity can promote amyloid-
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NMDA greatly increased Aβ40 levels (189% increase versus untreated control, \( p = 0.02 \)), and this effect was largely blocked by MK-801 (Fig. 3C). These findings support a role for NMDAR activity in the regulation of APP processing that shifts the balance from \( \alpha \)- to \( \beta \)-secretase processing.

We hypothesized that NMDARs affect APP processing by altering APP trafficking in neurons. To test this hypothesis, we measured cell surface levels of endogenous APP by biotin-labeling surface proteins on cultured hippocampal neurons, isolating these proteins with avidin beads, and immunoblotting for APP. Levels of cell surface APP were significantly decreased (by 87%, \( p < 0.01 \)) by NMDA compared with control or MK-801 treatments (Fig. 3, D and E). MK-801 again blocked the effects of NMDA on APP trafficking (resulting in an 11%, nonsignificant, decrease; Fig. 3, D and E). We also took an independent approach to measure the effect of NMDAR activity on APP trafficking, using live cell staining. Primary hippocampal neurons were co-transfected with NR1 and APP, and we measured cell surface levels of APP using live cell staining under nonpermeabilizing conditions. NR1 overexpression alone increased surface levels of APP compared with control vector (data not shown), allowing for more reliable assays of surface APP. Transfected cells were identified with GFP (Fig. 3F, left) and surface levels of APP with antibodies (right). We tested the effect of NMDAR activation over a brief period (20 min) by treating these cells with vehicle control (DMSO), NMDA (200 \( \mu M \)), or MK801 (10 \( \mu M \)) (Fig. 3F). NMDAR activation reduced cell surface levels of APP, whereas MK-801 treatment had no effect (Fig. 3F). Quantification of staining in neuronal processes showed a significant decrease in surface APP after NMDA (87% decrease versus untreated cells; \( n = 3; p < 0.01 \)), whereas MK-801 treatment had no effect. These data show not only that APP is found in the PSD but also that synaptic activity alters APP processing and trafficking.

Knockdown of APP Reduces NMDAR-mediated Current Responses—To examine the physiological function of APP, we decreased endogenous APP expression levels in primary neurons with RNAi and examined the effect on synapses using whole cell patch clamp recordings. This approach allowed us to specifically examine the effects of APP in the postsynaptic cell...
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without altering presynaptic APP. To determine the effectiveness of various APP RNAi, we transfected COS7 cells with rodent APP and RNAi constructs. Two APP RNAi constructs (numbers 7 and 14) in the pSuper vector (18) reduced transfected APP expression >90% (Fig. 4A); a third APP RNAi construct (number 37) had no effect. To test whether the RNAi was effective against endogenous APP in neurons, we co-transfected cultured primary hippocampal neurons with GFP (to identify transfected neurons) and either empty pSuper vector or APP RNAi-14 (Fig. 4B, left). After 3 days of expression, cultures were immunostained to detect endogenous APP. APP immunostaining was substantially reduced by APP RNAi-14, particularly in the neuronal processes, compared with control cells (Fig. 4B; 95% reduction versus pSuper control, p < 0.01) or cells transfected with the ineffective APP RNAi-37 (data not shown). Using transfected COS7 cells and primary neuronal cultures, we found that the APP siRNA also reduced levels of APP proteolytic fragments, secreted APP, and APP C-terminal fragment (data not shown). Thus, as expected, changes to APP also result in changes to APP fragments, such as secreted forms of APP, Aβ, and intracellular fragments.

To analyze changes in neuronal electrophysiology caused by altered APP levels, we initially examined mouse CGCs. These cultures provide a homogeneous neuronal population from which the total and synaptic receptor pools of receptors can be measured. With its small cell body size (only 3–4 μm in diameter) and simple dendritic arborization, the CGC allows determination of whole cell current response and synaptic current with much higher recording resolution compared with other types of cultured neurons. We transfected CGCs with APP RNAi-14 and GFP using calcium phosphate, as described (29). Fewer than 5% of neurons were transfected under these conditions, allowing us to focus on the effects of reducing APP in individual neurons, rather than neuronal networks. We obtained whole cell patch clamp recordings 24–48 h post-transfection from pairs of nearby APP RNAi-transfected and nontransfected control neurons, using a focal application of saturating doses of NMDA (200 μM). A second control group consisted of CGCs transfected with only GFP. The peak amplitude of current responses to this NMDA application represents the contribution of all functional surface NMDARs, including receptors localized to synaptic sites and those at extrasynaptic sites. These currents were subsequently normalized to cell capacitance (which is proportional to cell surface area) to generate current density values (the cultured CGCs had generally homogenous capacitances (5 ± 0.42 pF)). Neurons with decreased levels of APP exhibited reduced NMDA-evoked current densities compared with neurons transfected only with GFP (Fig. 4C). Quantification of results from neurons transfected with APP RNAi (n = 15) demonstrated a significant reduction in NMDA evoked current densities compared with GFP (n = 31) or nontransfected neighboring cells (n = 16) (70% reduction versus GFP control, p < 0.01) (Fig. 4D). These data demonstrate that endogenous APP affects normal neuronal NMDAR expression or function.

**APP Overexpression Increases NMDAR Currents**—Reducing APP levels decreased NMDAR current density in cultured neurons; we next examined whether transiently increasing APP levels had the opposite effect. Using the same protocol as above, we transfected CGCs with APP tagged on its C terminus with GFP. Neurons overexpressing APP exhibited significantly higher NMDA-evoked current densities than control untransfected neighboring cells or GFP-transfected cells (42% increase versus GFP control, p < 0.01) (Fig. 5, A and B). As in Fig. 4, APP siRNA reduced NMDA current density (Fig. 5, A and B). To examine the specificity of these effects, we analyzed inhibitory GABA_A receptors present in these CGCs. We found that there was no significant difference in response to a saturating dose of GABA (1 mM) observed between CGCs expressing APP-GFP and control cells transfected only with GFP (Fig. S1). Thus, the...
The effect of APP on NMDARs was not due to nonspecific effects on all neurotransmitter receptors. Because the hippocampus is one of the most vulnerable brain regions affected in AD, we tested whether the effects of APP on NMDARs were also observed in hippocampal neurons. Consistent with the results in CGCs, overexpression of APP caused a significant increase in NMDAR current density at DIV 11–13 hippocampal neurons (41% increase versus GFP control, p < 0.05) (Fig. 5, C and D). Knockdown of APP levels with RNAi again caused a reduction in NMDAR current density (22% decrease versus control), although the decrease did not reach significance in these experiments (p = 0.18).

The results from overexpression of APP were surprising because of reports demonstrating that the APP proteolytic product Aβ has a negative effect on glutamate receptors (4, 5, 7). To test whether Aβ produced in our cells could be having a confounding effect in our system, we compared Aβ levels in cultured medium from control CGCs and neurons overexpressing APP (from the low efficiency calcium phosphate transfections). We observed no significant difference in Aβ levels between these two groups (data not shown). We also transfected cells with APP-MV, a version of APP mutated at its β-cleavage site to prevent the production of Aβ (7). As in our experiments with wild-type APP, the β-site mutant APP caused increased NMDAR current density in both CGCs (32% increase, p < 0.01; Fig. 5, A and B) and hippocampal neurons (53% increase, p < 0.01; Fig. 5, C and D).

We also tested whether the direct or indirect addition of Aβ to the cultures had a similar effect as adding full-length APP. First, we transfected cells with a construct expressing the APP fragment C99, which can generate Aβ and which contains the intracellular domain of APP. In contrast to full-length APP, C99 significantly decreased NMDAR current density (36%, p < 0.05; Fig. 5, A and B). Second, we treated neurons with exogenous Aβ. We applied Aβ42 (2 μM) to cultured CGCs for 24–48 h and observed the expected significant decrease in whole cell NMDAR current densities (52% decrease versus control, p < 0.01) (Fig. S2). Therefore, we attribute the effect of APP-enhancing NMDAR whole cell current responses to full-length APP and not to any effects of the APP C-terminal fragment or the Aβ peptide.

APP Increases the Incorporation of the NR2B Subunit into Receptor Complexes—Given our findings that APP increased the current density of NMDARs, we considered the possibility that APP affected subunit composition of NMDARs, since NR2B subunit passes larger current than NR2A (13). To test this idea, we used the highly selective NR1/NR2B antagonist, CP101,606. Control GFP-transfected CGCs showed a modest but significant inhibition of NMDAR whole cell current density by CP101,606 (28% decrease versus vehicle, p < 0.01) (Fig. 6A). Consistent with the data in Fig. 5, APP-GFP-transfected cells showed an increase in NMDAR current density compared with GFP control. This enhanced current density was more sensitive to CP101,606 than control cells (46% reduction, p < 0.01) (Fig. 6A). Importantly, the whole cell currents from GFP- and from APP-GFP-transfected cells in the presence of CP101,606 were nearly identical, suggesting that the increase in NMDAR current seen with APP was due largely to the increase in CP101,606-sensitive receptors (i.e. NR1/NR2B heteromers). We performed similar experiments using another selective NR2B antagonist, ifenprodil (10 μM), and again found that APP selectively promoted functional expression of NR1/NR2B receptor complexes (data not shown). Thus, we conclude that APP specifically enhances the surface levels of NR2B-containing NMDARs.

To test these electrophysiological findings using an immunocytochemical assay, we conducted cell surface immunostaining...
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A

GFP

NMDA

NMDA+CP101,606

APP-GFP

NMDA

NMDA+CP101,606

100pA

2s

B

Current Density (pA/pF)

-CP

+CP

GFP

GFP

APP-GFP

APP-GFP

C

Tfx:

Total NR1

Surface NR1

NR1/Vector

NR1/APP

NR1/SiRNA APP

D

Tfx:

Total NR2A

Surface NR2A

NR2A/Vector

NR2A/APP

NR2A/SiRNA APP

E

Tfx:

Total NR2B

Surface NR2B

NR2B/Vector

NR2B/APP

NR2B/SiRNA APP

F

NMDAR surface intensity (% of control)

APP

SiRNA APP

G

NR2A

APP

SiRNA APP

H

NR2B

APP

SiRNA APP
of NMDARs in primary hippocampal neurons. To allow immunostaining of surface NMDAR subunits, we transfected neurons with NR1, NR2A, or NR2B tagged on their N-terminal (extracellular) domains with GFP. The presence of the GFP tag allowed imaging of surface receptors with a GFP antibody, since we were unable to identify reliable receptor subunit-specific antibodies for immunocytochemistry assays of endogenous NMDA receptor subunits. These GFP-tagged NMDAR subunits are known to express and traffic indistinguishably from their endogenous counterparts (17, 30, 31). The left panels define transfected cells; the middle panels show surface NMDA receptor subunits; and the right panels show a magnification of selected neuronal processes (Fig. 6, C–E). Compared with cells transfected with just the NMDAR subunit alone (defined as 100%), co-transfection with APP significantly increased surface levels of both NR1 (176% versus NR1 alone, p < 0.01; Fig. 6, C and F) and NR2B (147% versus NR2B alone, p = 0.02; Fig. 6, E and H) in dendritic processes. Overexpression of APP did not affect surface levels of NR2A (118% versus NR2A alone, p > 0.05; Fig. 6, D and G). In contrast, reduction of endogenous APP using RNAi significantly decreased dendritic surface staining of both NR1 (57% versus NR1 alone, p < 0.01; Fig. 6, C and F) and NR2B (60% versus NR2B alone, p < 0.01; Fig. 6, E and H) but not NR2A (93% versus NR2A alone, p > 0.05; Fig. 6, D and G). Because C99 decreased NMDAR current density, we tested whether it decreased surface levels of NR2B. We found that hippocampal neurons co-transfected with NR2B and APP again showed a significant increase in surface NR2B (135% of control; n = 7; p < 0.01) but that C99 showed a significant decrease in surface NR2B (76% of control; n = 7; p < 0.05). These data are consistent with our observation that C99 reduced NMDAR current density (Fig. 5).

Because a change in NMDAR surface expression could reflect changes in trafficking, we tested the effect of APP on internalization of NR2A or NR2B in hippocampal neurons. We co-transfected neurons with GFP-tagged NR2A or NR2B together with either empty vector, APP expression vector, or APP RNAi (Fig. 7, A and B, left). We then used an antibody uptake assay to measure internalization of NMDAR subunits (Fig. 7, A and B, right, at 30 min after labeling of surface NR2A or NR2B). Using this assay, co-transfection with APP significantly decreased endocytosis of NR2B (58% versus NR2B alone, p = 0.02) (Fig. 7, B and D). APP overexpression did not affect endocytosis of NR2A (90% versus NR2A alone, p > 0.05) (Fig. 7, A and C). In contrast, reduction of endogenous APP using RNAi significantly increased internalization of NR2B (139% versus NR2B alone, p < 0.05) (Fig. 7, B and D) but not NR2A (91% versus NR2A alone, p > 0.05) (Fig. 7, A and C). These findings are consistent with the electrophysiology data indicating that APP promoted surface levels of NR2B-containing receptor complexes preferentially.

APP Increases NMDA-mediated Synaptic Currents—Thus far, we have reported corresponding changes in NMDA-mediated whole cell currents (composed of both synaptic and extrasynaptic receptors) and surface levels of NMDAR subunits in cells with altered APP levels. We next tested whether APP affected NMDA receptors specifically within synapses by recording spontaneous miniature NMDA-mediated excitatory post synaptic currents (NMDA-mEPSCs) (22, 23, 29). Sample traces of NMDA-mEPSCs recorded in cultured CGCs at 7–8 days in vitro in the absence of Mg2+ under various conditions are illustrated (Fig. 8A). The peak amplitude of NMDA-mEPSCs was significantly increased by APP-GFP (129% versus GFP control, p < 0.05) and the APP-MV mutant (165% versus GFP control, p < 0.05) (Fig. 8C, left). These data indicate that APP causes an increased number of synaptic NMDA receptors in the postsynaptic densities or a change of biophysical channel properties, such as single-channel conductance or mean single-channel open time.

To address the possibility that the observed effects of APP might be due to changes in NMDAR single-channel properties, we took advantage of the fact that recordings at negative potentials are characterized by very low background noise in CGCs. This property allows for the observation and measurement of the amplitude of single-channel currents in the tails of synaptic responses (29). Using this methodology we observed no change in the single-channel NMDA current in neurons with altered APP levels (Fig. 8B) (3.2 ± 0.6 pA (mean ± S.D.) for control GFP-transfected cells; 3.5 ± 0.7 pA for APP-transfected cells; 3.3 ± 0.4 pA for APP siRNA-transfected cells). Thus, APP did not affect the single-channel conductance of postsynaptic NMDARs.

Finally, we observed that the weighted time constant of decay in APP-GFP-transfected cells (τw; Fig. 8C, middle) was significantly lengthened (for APP, 138% versus GFP control, p < 0.05; for APP-MV, 133% versus GFP control, p < 0.05), suggesting increased incorporation of NMDARs with slower deactivation kinetics. This property is consistent with a higher number of NR2B-containing receptors in the synapse. The frequency of NMDA-mEPSCs was not significantly affected by overexpression of APP or APP-MV (Fig. 8C, right). In cells transfected with APP siRNA, there was a significant reduction in NMDA-mEPSC peak amplitude (29% decrease versus GFP control, p < 0.05) and frequency (75% decrease versus GFP control, p < 0.05) (Fig. 8C, right). The decay time decreased in APP siRNA-transfected cells compared with GFP-transfected cells, although this did not reach significance (p = 0.11). Together, these data support a strong effect of APP on NMDAR function.
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within the synapse, increasing NMDAR numbers and favoring NMDAR with longer decay times.

DISCUSSION

Our data have defined a novel physiological function of APP in promoting NMDAR accumulation at postsynaptic sites. This conclusion is supported by several lines of evidence. APP co-localizes and co-fractionates with postsynaptic markers and co-precipitates with PSD proteins in the brain. Knockdown of endogenous APP decreased (and overexpression of exogenous APP increased) the NMDAR whole cell current density as well as NMDA-mEPSC peak amplitudes. These electrophysiological findings were consistent with our immunocytochemical studies showing that APP promoted the presence of NMDARs on the neuronal cell surface by inhibition of receptor endocytosis. The effect of APP overexpression was observed in both CGCs and hippocampal neurons, suggesting that this function may be a general feature of NMDAR-expressing cells. We observed the opposite effects when we added the C-terminal portion of APP (C99). These effects may be due to the local generation of the synaptotoxic Aβ fragment or to the competition of the transfected C99 with intracellular adaptor proteins that normally mediate the functions of endogenous APP. We conclude that the extracellular domain of APP is necessary for its effects on synaptic functions.

We confirmed and extended the earlier findings of Kamenetz et al. (7) that stimulating neurons with NMDA favored cleavage of APP by β-secretase over α-secretase. We observed decreased surface APP, decreased sAPP, and increased Aβ after NMDA treatment, each of which was blocked by MK-801. These observations are consistent with the hypothesis that synaptic activity promotes the endocytosis of surface APP, leading to its β-cleavage (32).

We and others (26, 27) have found that APP is also presynaptic. APP may form homodimers reaching across the synaptic cleft (33), thus allowing presynaptic APP to affect the function of postsynaptic APP. The experiments described here were aimed at studying changes only in postsynaptic APP. In other preliminary experiments, we used neurons from APP knock-out mice to test the effects of changing both pre- and postsynaptic APP (data not shown). However, we did not observe changes in NMDAR current density in APP knock-out CGCs. We hypothesize that this result could be because eliminating APP both pre- and postsynaptically results in a more complex phenotype. Alternatively, functional redundancy with APP family members or developmental compensation may limit the usefulness of knock-out cultures. Presynaptic APP may have independent functions on synaptic function by altering presynaptic transporters (34) or vesicle release (35).

Overexpression of APP caused an enhancement of NMDA-mEPSC amplitudes in CGCs, indicative of increased spontaneous NMDAR activity in the cultures, and APP siRNA had the opposite effect. APP siRNA also caused a dramatic reduction in NMDA-mEPSC frequency. It may be that when APP function is lost acutely, NMDAR insertion at the synapse is compromised to the extent that many excitatory synapses become function-

FIGURE 7. APP inhibits NR2B receptor internalization in cultured hippocampal neurons. A and B, cultured hippocampal neurons were transfected at DIV 12 with constructs as indicated, and internalization of NR2 subunits was measured at DIV 18 by immunofluorescence of live cells. Left, total GFP; right, internalization of NR2A (A) or NR2B (B). C and D, quantification of NR2 internalization in neuronal processes. C, overexpression of APP or knockdown of APP did not alter internalization of NR2A (90 ± 19 or 91 ± 32%, respectively, versus control; n = 7; p > 0.05). D, APP significantly decreased NR2B internalization (58 ± 16% versus control; n = 14; *, p = 0.02), and APP siRNA significantly increased NR2B internalization (139 ± 27% versus control; n = 14; *, p < 0.05). All data are shown as mean ± S.E.
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A role for APP in synaptic plasticity is also suggested by its specific recruitment of NR2B-containing NMDARs. APP increased the decay time of NMDA-mEPSCs, enhanced surface expression, and suppressed internalization specifically of NR2B containing receptors. NR2B-containing receptors have several distinct properties. NR2B is expressed earlier in development than NR2A (14, 36, 37) and has a longer mean open time (13).

Although there is some debate regarding the precise roles of NR2A versus NR2B in synaptic plasticity, both NR2A and NR2B appear to be able to participate in long term potentiation and long term depression (38–40). However, NR2B contacts distinct sets of signaling proteins (41–43). It is possible that APP preferentially affects NR2B-containing receptors because the slower deactivation kinetics conferred by NR2B allows for greater calcium influx, which could favor heightened synaptic activity in response to neuronal damage or during learning.

We do not know the mechanism for how APP would preferentially affect NR2B, and we did not observe a specific interaction between APP and NR2B in immunoprecipitation experiments (data not shown). Our data demonstrate that full-length APP increases surface NR2B and decreases its internalization.

The role of APP in promoting synaptic recruitment of NMDARs is in contrast to the effects of Aβ on synapses. A number of recent studies (4, 5, 7) have found that Aβ (and specifically soluble, oligomeric Aβ) is synaptotoxic, resulting in decreased levels of AMPARs and NMDARs at synapses in primary neurons. Aβ also results in decreased long term potentiation (4) and decreased spine number in transgenic models of AD (44). We confirmed in our system that Aβ(1–42) decreased NMDA current densities, whereas full-length APP increased NMDAR currents in the same cell preparations. This differential effect is likely to reflect distinct roles of full-length APP and Aβ as well as the different levels of Aβ in these two conditions. For analysis of cells with overexpressed APP, we used calcium phosphate transfections (with low transfection efficiency) to limit the amount of excess Aβ that could be produced. Indeed, we observed no changes in synaptic transmission in neighboring neurons near APP-transfected cells, suggesting that low levels of Aβ secreted into the conditioned media from transfected cells were not sufficient to affect normal synaptic transmission or counteract the positive effect of APP on NMDAR recruitment. Furthermore, we showed that APP mutated at the β-secretase site showed enhancement of NMDAR whole cell current densities comparable with that seen with wild-type APP, demonstrating unequivocally that neither β-cleavage of APP nor the generation of Aβ was responsible for this effect. Thus, we propose that full-length APP promotes synaptic transmission, but its proteolytic product Aβ is synaptotoxic.

Taken together, our data suggest a model that ties together the functions of APP in physiological and pathophysiological conditions. APP is transported in both axonal and dendritic compartments (45) and localized to both pre- and postsynaptic regions (26, 27). Following many types of neuronal damage, levels of APP are increased (46, 47). Our data suggest that this increase may act as a mechanism to stabilize or strengthen synapses by recruitment of NMDARs or enhance plasticity by specifically increasing NR2B content. Aβ is also produced in response to damage; this production may be a result of the synaptic APP undergoing endocytosis and proteolysis in conjunction with heightened NMDAR activity. In turn, the loss of the stabilizing effect of full-length APP coupled with the synaptotoxic effect of Aβ could act as a physiological brake mechanism to down-regulate glutamatergic transmission (e.g. to combat excitotoxicity if synaptic activity levels become excessive). The synaptotoxic effects of Aβ in AD could be an aberrant or exaggerated form of this protective negative feedback response, thus explaining the loss of NMDARs during AD as well as the beneficial effects of the NMDAR antagonist memantine for AD patients. Further elucidation of APP function and trafficking in relation to NMDARs may contribute to our understanding of how the Aβ peptide is generated in normal and pathological states and inform ideas about how to help prevent its production in AD.

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