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Beta amyloid-independent role of amyloid precursor protein in generation and maintenance of dendritic spines

Kea Joo Lee^{1,†}, Charbel E.-H. Moussa^{2,†}, Yeunkum Lee¹, Youme Sung³, Brian W. Howell⁵, Raymond Scott Turner³, Daniel T. S. Pak^{1,*}, and Hyang-Sook Hoe^{3,4,*}

¹Department of Pharmacology, Georgetown University Medical Center, 3970 Reservoir Road NW, Washington, DC 20057-1464, USA

²Department of Biochemistry, Georgetown University Medical Center, 3970 Reservoir Road NW, Washington, DC 20057-1464, USA

³Department of Neurology, Georgetown University Medical Center, 3970 Reservoir Road NW, Washington, DC 20057-1464, USA

⁴Department of Neuroscience, Georgetown University Medical Center, 3970 Reservoir Road NW, Washington, DC 20057-1464, USA

⁵Department of Neuroscience and Physiology, SUNY Upstate Medical University, 750 E Adams St, Syracuse NY, 13210, USA

Abstract

Synapse loss induced by amyloid beta (A β) is thought to be a primary contributor to cognitive decline in Alzheimer's disease. A β is generated by proteolysis of amyloid precursor protein (APP), a synaptic receptor whose physiological function remains unclear. In the present study, we investigated the role of APP in dendritic spine formation, which is known to be important for learning and memory. We found that overexpression of APP increased spine number, whereas knockdown of APP reduced spine density in cultured hippocampal neurons. This spine-promoting effect of APP required both the extracellular and intracellular domains of APP, and was accompanied by specific upregulation of the GluR2, but not the GluR1, subunit of AMPA receptors. In an *in vivo* experiment, we found that cortical layers II/III and hippocampal CA1 pyramidal neurons in 1 year-old APP-deficient mice had fewer and shorter dendritic spines than wild-type littermates. In contrast, transgenic mice overexpressing mutant APP exhibited increased spine density compared to control animals, though only at a young age prior to overaccumulation of soluble amyloid. Additionally, increased glutamate synthesis was observed in young APP transgenic brains, whereas glutamate levels were decreased and GABA levels were increased in APP-deficient mice. These results demonstrate that APP is important for promoting spine formation and is required for proper spine development.

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*Co-corresponding authors: Hyang-Sook Hoe, Ph.D. Department of Neuroscience, Department of Neurology, Georgetown University Medical Center, 3970 Reservoir Road NW, Washington, DC, 20057-1464, USA. Tel. 1-202-687-8673; Fax 202-687-0617; hh69@georgetown.edu, Daniel T.S. Pak, Ph.D. Department of Pharmacology, Georgetown University Medical School, 3900 Reservoir Road NW, Washington, DC 20057, USA. Tel. 1-202-687-8750; Fax 1-202-687-8825; dtp6@georgetown.edu.

[†]These authors contributed equally to this work.

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Keywords

APP; GluR2; AMPA receptor; amyloid beta; glutamate; Alzheimer's Disease

Alzheimer's disease (AD) is an age-related neurodegenerative disease and the most common cause of dementia in the elderly. Clinically, AD is characterized by progressive deterioration of cognitive function and memory. The size of specific brain regions (hippocampus, entorhinal cortex, and amygdale) involved in learning, memory, and emotional behaviors is reduced in AD patients (Hyman et al., 1984, LeDoux, 1993, Cahill et al., 1995). AD pathological hallmarks consist of intracellular extracellular amyloid plaques and neurofibrillary tangles composed of hyperphosphorylated tau protein (Huang and Jiang, 2009). Proteolytic processing of the synaptic protein APP produces a 40 or 42 amino acid protein fragment, A β , which is the chief component of amyloid plaques. Intense research has focused on the detrimental effects of A β on cognition, yet a physiological function for APP remains elusive.

APP is a transmembrane glycoprotein and synaptic adhesion molecule that is a member of a family of proteins including APP-like proteins APLP1 and APLP2. Mice lacking all three APP family members demonstrate neuronal migration abnormalities in the brain and die at various stages of development. With in utero APP knockdown, migration defects are also observed due to inhibition of cortical plate entry of neuronal precursor cells, while APP overexpression causes migration of cells to overshoot past the cortical plate. Therefore, normal APP levels are likely necessary for appropriate neuronal positioning (Young-Pearse et al., 2007).

Studies have suggested an evolutionarily conserved role for APP in synapse formation or stability. APP is preferentially localized to synaptic puncta in both peripheral and central synapses. Its expression peaks at the developmental period in mammals in which the most rapid cortical synaptogenesis occurs, around the second postnatal week. A recent study showed that the extracellular domain of APP promotes synapse formation using heterologous co-culture system, suggesting that transsynaptic interactions between pre- and postsynaptic APP promote the adhesion of synapses (Wang et al., 2009).

Overexpression of the APP homolog in *Drosophila* promotes synapse differentiation (Torroja et al., 1999), while its elimination prevents cell adhesion molecule-stimulated synapse formation (Ashley et al., 2005). Expression of APP in heterologous cells also promotes synaptogenesis in contacting axons by mixed-culture assay (Wang et al., 2009). In rodents, APP expression peaks around the second postnatal week, during robust synaptogenesis (Loffler and Huber, 1992). Furthermore, mice deficient in APP or the related family member APP-like protein 2 (APLP2) show defective apposition of presynaptic and postsynaptic proteins at neuromuscular junctions (Wang et al., 2005), while APP/APLP2 double knock-outs (KO) exhibit presynaptic deficits in ganglion inter-neuronal synapses (Yang et al., 2005). Similarly, small interfering RNA (RNAi) against APP causes impaired synaptic activity *in vivo* (Herard et al., 2006). However, other studies of adult APP KO mice revealed no significant alterations in synaptic strength using extracellular stimulation electrodes to induce synaptic vesicle release (Dawson et al., 1999), no change in synaptic protein profiles (Soba et al., 2005), and even *more* excitatory synapses than wild-type littermates (Priller et al., 2005). Thus, while several lines of evidence support the roles of APP family members in the formation of proper neuronal connections, there is no consensus on the precise nature of these functions.

Here, we systematically addressed the role of APP in dendritic spine formation in cortical layers II/III and the hippocampal CA1 region *in vitro* and *in vivo*, and at different developmental states. Interestingly, we found that APP promotes spine formation with increased clustering of pre- and post-synaptic proteins. Additionally, we observed that APP increases cell surface levels of AMPA receptor subunit GluR2, but not GluR1. Consistent with these observations, we found that glutamate levels were decreased in APP knockout mice, whereas glutamate levels were significantly increased in 1-month-old APP overexpressed mice. These findings suggest that increased glutamate synthesis may accompany increased neurotransmitter release into the synapse which promotes spine formation.

Experimental Procedures

Animals

All animals were maintained according to protocols approved by the Georgetown University Animal Welfare and Use Committee. Wild-type C57BL/6J and APP knock-out mice (B6.129S7-APP^{tm1DB0/J}) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). APP transgenic mice (Tg2576) (129S6.Cg-Tg(APP^{SWE})2576Kha N20) were obtained from Taconic (Germantown, NY, USA).

Constructs

The pEGFP construct was commercially obtained (Clontech, Mountain View, CA, USA). All constructs utilized APP-770 isoform unless otherwise noted. For RNA interference, shRNA against APP (#14, targeting nt485-503 of rat APP-770) cloned into the pLentiLox3.7 (pLL3.7) vector (Rubinson et al., 2003) has been described (Hoe et al., 2009). This shRNA target sequence is not found in other APP family members or in human APP. We produced full-length human APP with a C-terminal myc tag and also produced an APP (M671V) construct using site-directed mutagenesis to convert the methionine residue to valine at amino acid 671 of APP. We generated N-terminal signal peptide and C-terminal myc-tagged deletion constructs of APP as follows: APP Δ E1 (residues 262aa–770aa), Δ E1E2 (residues 411aa–770aa), and β -CTF (residues 671aa–770aa).

Primary neuron culture and immunostaining

Primary hippocampal neurons from E19 Sprague–Dawley rats were cultured at 150 cells/mm² as described (Pak et al., 2001). Neurons were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) or calcium phosphate precipitation. The following antibodies were used: mouse anti-GFP (Qbiogene MP Biomedicals, Irvine, CA, USA), rabbit anti-GFP (Invitrogen), rabbit anti-GluR1 (EMD Calbiochem, Gibbstown, NJ, USA), mouse anti-GluR2 (BD Pharmingen, San Jose, CA, USA), mouse anti-PSD-95 (NeuroMabs, Davis, CA, USA), mouse anti-Bassoon (Assay Designs, Ann Arbor, MI, USA), rabbit APP N-terminal (Sigma-Aldrich, St. Louis, MO, USA), and mouse anti-c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunostaining of surface GluR1 and GluR2 in hippocampal neurons was performed as described (Hoe et al., 2007). Briefly, live neurons were incubated with N-terminal GluR1 or GluR2 antibodies (10 μ g/ml in conditioned medium) for 10 min, then briefly fixed in 4% paraformaldehyde (non-permeabilizing conditions). Surface-labeled GluR1 or GluR2 was detected with Alexa fluor-555 secondary antibodies. Cells were then permeabilized in methanol (–20°C, 90 sec), and incubated with anti-GFP antibody to identify transfected neurons.

Golgi staining and morphological analysis of dendritic spines

To analyze dendritic spine morphology in brain, FD Rapid GolgiStain Kit (FD NeuroTechnologies, Ellicott City, MD, USA) was used. Dissected mouse brains were immersed in Solution A and B for 2 weeks at room temperature and transferred into Solution C for 24 hours at 4°C. Brains were sliced using a VT1000S Vibratome (Leica, Bannockburn, IL, USA) at 150 µm thickness. Dendritic images were acquired by Axioplan 2 (Zeiss, Oberkochen, Germany) under brightfield microscopy. Spine width, length, and linear density were measured using Scion image software (Scion Corporation, Frederick, MD, USA). Cultured hippocampal neuron images were acquired by LSM 510 laser scanning confocal microscope (Zeiss). Confocal z-series image stacks encompassing entire dendrite segments were analyzed using MetaMorph software (Universal Imaging Corporation, Downingtown, PA, USA). Spines from 0.2 to 2 µm in length were included for analysis. All morphological analysis was done blind to genotypes or experimental conditions.

NMR spectroscopy of brain extracts

Animals fasted overnight with free access to tap water were injected intraperitoneally with [1-¹³C]glucose solution (0.5 mol/L) over 10 seconds (0.3 ml/25–30g body weight; 200 mg/kg). After 45 minutes animals were sacrificed by cervical dislocation and brains immediately homogenized in 6% ice-cold perchloric acid, 50 mM NaH₂PO₄. Homogenates were centrifuged 15 min at 4°C and 4500 rpm, and supernatants neutralized to pH 7.2 with KOH (pellets retained for protein estimation), then centrifuged 15 min at 4°C and 4500 rpm to remove salts, lyophilized and stored at –20°C. Extracts were resuspended in 0.65ml D₂O containing 2 mM sodium [¹³C]formate as internal intensity and chemical shift reference (δ 171.8). Metabolite pool size was identified on ¹H {¹³C-decoupled} NMR spectra. Peak areas were adjusted for nuclear Overhauser effect, saturation and natural abundance effects and quantified by reference to [¹³C] formate. Metabolite pool sizes were determined by integration of resonances in fully relaxed 400 MHz {¹³C-decoupled} ¹H spectra using NAA as internal intensity reference. Incorporation of ¹³C into isotopomers was measured in reference to [¹³C]formate. All data were collected on a 9.7 Tesla Varian Spectrometer with dual ¹³C/¹H probe. {¹³C-decoupled}-¹H spectra were acquired with 3000 scans, pulse width 45°, relaxation delay 1 second, line broadening 0.5 Hz, acquired data points 13.132 and transformation size 32K at room temperature. {¹H-decoupled}-¹³C spectra were acquired with 24000 scans and 31875 data points.

Statistical analyses

All values were expressed as mean ± SEM and Student's t-test was used for two-group comparisons and one-way ANOVA with Tukey's *post hoc* test for multiple group comparisons, unless stated otherwise. Statistical significance was determined at *p*<0.05. All experiments were conducted a minimum of three times unless otherwise noted.

Results

APP promotes dendritic spine formation

To investigate the role of APP in spine formation, we transfected primary rat hippocampal neurons at days *in vitro* (DIV) 21 with EGFP vector and expression plasmids encoding either wild-type APP, APP (M671V) mutant preventing cleavage by β-secretase (Citron et al., 1996), or with short hairpin RNA (shRNA) against endogenous APP (APP-shRNA #14) in vector pLL3.7. After 3 days, dendritic spine density was analyzed using confocal microscopy. Neurons overexpressing APP or APP (M671V) had significantly more spines along secondary dendrites, compared to neurons transfected with GFP control (Fig. 1A–C and G). In contrast, knockdown of endogenous APP decreased spine density by

approximately 30% (Fig. 1D, E and H). To demonstrate RNAi specificity, we coexpressed a shRNA-resistant rescue construct of APP (human APP) that completely prevented the spine decrease seen with shRNA knockdown of endogenous rat APP (Fig. 1F and H). To correlate APP expression level with spine number, we performed quantitative immunocytochemistry using APP antibodies in untransfected control, APP overexpressing, and APP knockdown neurons (Supplemental Fig. 1A, B). We observed a remarkably linear relationship between APP levels and number of spines (Supplemental Fig. 1C). However, spine density does not extrapolate to zero along with APP levels. These observations strongly support the idea that APP is fundamentally coupled to the mechanisms that determine spine density, but may not be absolutely essential for spine formation.

Intracellular and extracellular domains of APP are involved in spine regulation

To determine which domain of APP is essential for spine regulation, we generated several myc-tagged APP deletion constructs (Fig. 1I), which were expressed at comparable levels in heterologous cells (Fig. 1J). Primary hippocampal neurons (DIV21) were co-transfected with EGFP and APP deletion constructs (Δ E1, Δ E1E2, and β -CTF) for 3 days. Quantitative spine analysis showed that none of the deletion mutants were capable of increasing spine density (Fig. 1K–N and O). Indeed, both the APP β -CTF and the deletion construct lacking the conserved extracellular E1 and E2 domains (Δ E1E2) caused significantly decreased spine density (Fig. 1O), suggestive of a dominant-negative effect.

To further test whether deletion constructs of APP act in a dominant negative fashion, we measured their ability to block Reelin signaling elicited by activation of the full-length APP in COS-7 cells. We and others have previously shown that Reelin induces Dab1 tyrosine phosphorylation via the Fyn tyrosine kinase (Howell et al., 1999, Arnaud et al., 2003, Hoe et al., 2006a, Hoe et al., 2006b, Hoe et al., 2008). Further, we showed that Reelin increases APP-Dab1 interaction, leading to greater Dab1 tyrosine phosphorylation by constitutively active (Fyn-CA) (Hoe et al., 2006a, Hoe et al., 2008).

Using this system, we verified that Reelin treatment increases Dab1 phosphorylation in the presence of full-length APP and Fyn-CA (Supplemental Fig. 2). However, co-transfection of full-length APP with either Δ E1 or β -CTF significantly attenuated Reelin-induced Dab1 tyrosine phosphorylation by Fyn-CA (Supplemental Fig. 2). Our interpretation of these results is that Δ E1 and β -CTF, neither of which interact with Reelin, can still interact with and sequester the cytoplasmic proteins Dab1 and Fyn from full-length APP. These data indicate that APP deletion constructs can act in a dominant negative manner with regard to APP signaling.

Our recent study showed that interaction of Reelin with the E1 domain of APP is important for neurite outgrowth in developing neurons (Hoe et al., 2009), and APP and Reelin protein levels are highest at postnatal days 1 and 10, periods of high synaptogenesis (Hoe et al., 2009). Therefore, we examined whether APP and APP deletion constructs may alter spine formation in younger neurons. Interestingly, all deletion constructs significantly reduced the number of spines along the dendrites at this age (Fig. 2A–E). These data suggest that both extracellular and intracellular domains of APP are important for proper spine formation.

APP affects clustering of synaptic proteins

To corroborate these spine analyses, we tested whether APP affected the accumulation of endogenous synaptic proteins. We conducted immunostaining of PSD-95 as a postsynaptic marker and Bassoon as a presynaptic marker in primary hippocampal neuronal cells with overexpressed APP or knockdown of APP. APP or APP (M671V) overexpression significantly increased puncta density of PSD-95 (Fig. 3B, C and G) and Bassoon (Fig. 3J, K

and O). Conversely, knockdown of APP reduced the number of PSD-95 (Fig. 3E and H) and Bassoon (Fig. 3M and P) dendritic puncta, effects fully reversed by the human APP rescue construct (Fig. 3F, H, N, and P). Together, these results indicate that transient changes in APP expression can control excitatory synapse number in cultured hippocampal neurons.

APP regulates the expression of GluR2 subunits of AMPA receptors but not GluR1

To further analyze the actions of APP in spine formation, we investigated the cell surface and total levels of AMPA receptor subunits (AMPA receptors), because the abundance of synaptic AMPARs correlates with spine density as well as spine head size in hippocampal neurons (Matsuzaki et al., 2001, Passafaro et al., 2003). Furthermore, AMPAR levels are reduced in 6-month-old double knock-in mice carrying mutated human APP and presenilin-1 genes (Chang et al., 2006). These findings imply that the effect on dendritic spines by APP may induce changes in AMPAR expression.

To test this idea, we transfected hippocampal neurons with EGFP and either APP or APP-shRNA constructs. After 3 days, we performed live cell-surface staining of AMPA receptor subunit GluR2 under non-permeabilizing conditions and total GluR2 under permeabilizing conditions (Fig. 4A–N). APP overexpression specifically and significantly increased the fluorescent intensity of surface and total GluR2 (Fig. 4B and I, quantified in F and M). In contrast, knockdown of endogenous APP led to a specific decrease in surface and total GluR2 levels (Fig. 4D and K, quantified in G and N). As before, the effects of APP-shRNA could be fully rescued by coexpression of human APP (Fig. 4E and L, quantified in G and N).

Next, we transfected each APP deletion construct into hippocampal neurons (DIV14), and cell-surface and total GluR2 levels were measured under non-permeabilizing and permeabilizing conditions, respectively (Fig. 4O–X). All deletion constructs significantly decreased fluorescent intensity of both surface and total GluR2 compared to a control transfected GFP vector (Fig. 4O–R and T–W, quantified in S and X). These data indicate that APP-induced increases in spine number may be accompanied by enrichment in GluR2 receptors.

To further test whether APP could affect other AMPAR subunits such as GluR1, we transfected hippocampal neurons with EGFP and either APP or APP-shRNA constructs. After 3 days, we performed live cell-surface staining of GluR1 under non-permeabilizing conditions and total GluR1 under permeabilizing conditions (Fig. 5). APP overexpression did not alter the fluorescent intensity of surface and total GluR1 (Fig. 5B, I, F, and M). Furthermore, knockdown of endogenous APP did not alter cell surface levels of GluR1 or total GluR1 (Fig. 5D, K, G, and N). These data suggest that APP regulates certain AMPAR subunits, specifically GluR2, while it demonstrates no effects on GluR1.

Spine morphometry in APP-deficient mice

To examine whether APP also promotes spine formation *in vivo*, we carried out quantitative analysis of spine density in adult APP-deficient mice using Golgi staining. We focused on pyramidal neurons in cortical layers II/III and hippocampal CA1 area, because extracellular amyloid deposits were frequently observed in these brain regions of AD animal models (Hsiao, 1998), and because decreased spine numbers were reported in cortical pyramidal neurons of AD patients and old age AD model mice (Catala et al., 1988, Spires et al., 2005, Spires-Jones et al., 2007). We found that compared with wild-type (WT) littermates, mean spine density was significantly decreased in APP knockout (KO) mice in the hippocampal CA1 region (Fig. 6B, quantified in C). We also measured spine length and head width to compare the structure of individual spines between WT and APP-deficient mice in the

hippocampal CA1 region. Cumulative distribution plots revealed that APP-deficient mice had much shorter spines than WT animals (Fig. 6D), without change in spine head width (Supplemental Fig. 3B). Similarly, compared to WT littermates, APP heterozygous mice carrying a single copy of APP exhibited decreased dendritic spine density, and APP KO mice demonstrated a further decrease in both apical oblique and basal dendrites (see diagram in Fig. 6A) of cortical pyramidal neurons (Fig. 6E; apical and basal data pooled for quantification in F). Cumulative distribution plots revealed that APP-deficient mice had much shorter cortical spines than WT mice (Fig. 6G), without changing spine head width (Supplemental Fig. 3A). Thus, APP is required to attain the appropriate number of spines and complete the morphogenesis of individual spines in the intact brain.

Spine morphometry in APP-transgenic mice

To complement the above studies using reduced APP levels, we examined the effect of APP overexpression on spine density and morphology. We used 1-month and 1-year-old APP transgenic mice (TG) overexpressing the Swedish mutant form of human APP (APP_{SWE}; Tg2576). Since this transgenic line develops depositions of dense amyloid plaques in cortex, subiculum, and presubiculum as a function of age (Hsiao et al., 1996), we first analyzed dendritic spine density in adult (12-month-old) animals. Consistent with previous studies (Lanz et al., 2003), we found that adult APP TG mice had significantly fewer dendritic spines on pyramidal neurons in both cortex and hippocampus compared to WT littermates (Fig. 7B and D). This observation was potentially due to a noxious effect of extracellular soluble amyloid oligomers on spines, as reduced spine density in adult TG mice correlated with ~93% increased levels of soluble A β (1–40) from TG brain lysates (Supplemental Fig. 4). In contrast to APP KO mice, there was no significant difference in spine length and width between adult APP TG and WT animals in cortical layers II/III and hippocampal neurons (Fig. 7C and E and Supplemental Fig. 3C–D), suggesting that the development of individual spines was normal in adult APP TG mice.

Because of the potentially confounding issue of A β on spine density, we examined the effect of APP_{SWE} expression on spines in young (1-month-old) APP TG mice, an age at which TG mice do not show measurable A β overaccumulation (Supplemental Fig. 4). Interestingly, young APP TG mice had significantly *increased* numbers of spines on both cortical layer II/III and hippocampal CA1 pyramidal neurons compared to WT littermates (Fig. 7G and I). Analysis of spine head width and length showed that young TG mice had slightly but significantly longer and smaller spines in the hippocampus CA1 region compared to WT (Fig. 7H and Supplemental Fig. 3F), possibly reflecting ongoing spine formation. However, we could not detect a difference in spine morphology of cortical pyramidal neurons in young TG mice (Fig. 7J and Supplemental Fig. 3E). Together, these data suggest that APP is sufficient to promote increased spine density *in vivo*.

NMR analysis of amino acid transmitters in APP knockout and transgenic mice brains

In order to determine whether the effects of APP on dendritic spines and synapses were reflected in excitatory (glutamate) and inhibitory (GABA) neurotransmitter balance, we performed high frequency {¹H-decoupled}¹³C NMR analysis of brain extracts. The results showed no significant effects on metabolic flux between APP KO and control mice pertaining to glutamate, GABA, aspartate, glutamine and lactate (Fig. 8A), suggesting no difference in metabolic activities between KO and control mice. However, percentage of ¹³C incorporation from [1-¹³C] glucose was significantly increased into glutamate C2 (30%) and C4 (30%) positions compared to control levels (Fig. 8B), as was incorporation into GABA C2 (100%) and glutamine C4 (130%) isotopomers. A significant increase in the fractional enrichment of aspartate C2 (100%) and C3 (100%) was observed in comparison to control. Notably, the pool size of glutamate was significantly decreased in APP KO mice (40%)

while GABA was significantly increased (25%) compared to control (Fig. 8C), suggesting an imbalance between the levels of excitatory and inhibitory amino acids in these mice.

APP TG mice showed a significant increase in metabolic flux into both glutamate C2 (~26%) and C4 (43%) compared to control (Fig. 8D). This significant increase in metabolic flux was also observed in Asp C2 (28%). No changes in the fractional enrichment of the isotopomers were observed between APP TG and control mice (Fig. 8E), except for GABA, glutamine, and aspartate. However, a significant increase in the pool size of glutamate (105%) and aspartate (38%) was observed in APP TG compared to control mice (Fig. 8F), reflecting a substantial increase of excitatory amino acid synthesis in TG animals.

Discussion

In this study, we used a combination of *in vitro* and *in vivo* methods to define a novel physiological role for APP in promoting dendritic spine development. In cultured hippocampal neurons, full-length APP overexpression caused higher spine density (but not size), while knockdown of APP produced the opposite result. Somewhat unexpectedly, we observed that APP levels were tightly correlated with spine density, obeying a nearly perfect linear relationship. These data imply that APP protein levels are a key determinant of spine number.

The spine-promoting effect of APP was also observed with the APP (M671V) mutant, and thus is not dependent on cleavage of APP by β -secretase. Instead, the spine-promoting effect was dependent on the E1 extracellular domain of APP, which is known to bind to the ligand Reelin. These findings are also supported by a recent study showing that Reelin signaling promotes spine development in hippocampal neurons (Niu et al., 2008). In younger cultured neurons, both E1 and E2 domains appeared to be important for proper spine formation, as expression of APP constructs lacking these domains caused dominant-negative decreases in spine density. Because the truncated APP β -CTF construct also decreased spine density, we propose that coupling of extracellular ligands to intracellular effector molecules by full-length APP is a critical feature of its spine-promoting function, consistent with the ability of APP deletion constructs to block Reelin-induced signaling by full-length APP in heterologous cells. We also note that it is formally possible that APP stabilizes spines that are undergoing continuous turnover rather than causing *de novo* spine formation.

Increased spine number by APP expression was accompanied by increased numbers of excitatory synapses, as judged by the synaptic markers PSD-95 and Bassoon. Interestingly, we also observed increased total and surface GluR2/3 in APP overexpressing neurons, without any apparent change in GluR1. We speculate that this observation may be related to the proposed role of GluR2 subunit in facilitating dendritic spine formation (Passafaro et al., 2003). However, it is currently unknown how APP specifically regulates GluR2 expression levels, or whether increases in GluR2 mediate the effects of APP on spine density. Further study will be required to investigate these issues.

Importantly, we examined the role of APP in dendritic spine regulation *in vivo* using both gain-of-function and loss-of-function mouse models. This analysis clearly demonstrated loss of dendritic spines in both cortex and hippocampus in a manner dependent on APP gene dosage, with reduced spine density in APP HT mice and further reductions in APP KO animals. Furthermore, the remaining spines in KO brains were much shorter than WT control spines. Thus, APP is required for proper spine morphogenesis as well as number.

On the other hand, the effect of APP overexpression on dendritic spines was highly dependent on the age of transgenic mice examined. In young APP TG mice, both cortical and hippocampal pyramidal neurons showed increased numbers of spines having relatively

normal structure, suggesting that APP is sufficient to develop overabundant spines. In older animals, however, APP TG mice displayed significant spine loss, likely due to age-dependent accumulation of amyloid beta that may overcome the positive influence of full-length APP on spines. This developmental dependence in the effect of APP on dendritic spines may help reconcile apparent discrepancies in the literature regarding the role of APP in spine and synapse formation. For example, a recent study showed that APP knockout mice show a two-fold higher density of dendritic spines in the cerebral cortex (Bittner et al., 2009). The reason for these conflicting results may be due to the difference in age of the mice, imaging methodology, or brain region examined (we used 12 month animals, Golgi staining, and analyzed cortical pyramidal neurons in layer II/III as well as CA1 region of hippocampus, whereas Bittner et. al. used 4–6 month mice, two-photon confocal microscopy live imaging, and quantified spines in cortical pyramidal layer I/II). Clearly, APP function at synapses is complex and may not be uniform across all synapses. Further systematic analyses of various types of synapses in APP KO mice will be necessary to resolve these discrepancies.

Finally, because of the effects of APP on excitatory synapse number, we examined the metabolism of amino acid neurotransmitters in APP KO and TG mice. In APP KO mice, glutamate levels (pool size) were decreased, whereas the level of glutamate was increased by APP overexpression, suggesting that APP may promote glutamate synthesis in the brain. The significant increase in glutamate in young APP TG mice is consistent with the changes observed in GluR2/3 receptor levels, suggesting that increased glutamate synthesis could accompany increased neurotransmitter release into the synapse and promotion of spine formation. Upregulation of bulk excitatory neurotransmitter levels by APP is suggestive of a fairly substantial increase in synapse number or activity. These results carry the implication that APP control of dendritic spines may not be limited to a small number of synapses in restricted brain regions, but rather affects wholesale excitatory synaptic transmission. Additionally, we speculate that the decreased level of total glutamate in the APP KO mice could contribute to (or reflect) decreased learning ability and neural plasticity, both of which have been observed in these mice.

Conclusion

Taken together, our results demonstrate for the first time that APP promotes dendritic spine formation and is required to maintain appropriate spine numbers *in vitro* and *in vivo*. By providing a better understanding of the physiological synaptic roles of APP, this study may provide fundamental insights into the synaptic deficits observed in AD pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

AD	Alzheimer's Disease
AMPA	α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
APLP	APP-like protein
APP	amyloid precursor protein
APP_{SWE}	Swedish mutant of amyloid precursor protein
CA1	cornu ammonis area 1

CTF	C-terminal fragment of APP
DIV	days in vitro
EGFP	enhanced green fluorescent protein
GABA	gamma-aminobutyric acid
HT	heterozygote
KO	knock-out
RNAi	ribonucleic acid interference
sAPP	secreted APP
shRNA	short hairpin ribonucleic acid
PSD-95	postsynaptic density-95
TG	transgenic
WT	wild-type

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Figure 1.

APP promotes dendritic spine formation. A–F. Cultured hippocampal neurons (DIV21) were transfected with expression vectors encoding EGFP and empty vector, APP, APP (M671V), or shRNA against APP as indicated. To rescue the RNAi effect, a shRNA-resistant form of APP (human APP) was coexpressed with APP shRNA. G. Quantification of spine density (10 neurons/group, ** $p < 0.01$; *** $p < 0.001$). H. Quantification of spine density (10 neurons/group, * $p < 0.05$). I. Serial APP deletion constructs with a C-terminal myc-tag were generated containing the following amino acid residues: 265–770 (ΔE1), 591–770 (ΔE1E2), and 671–770 (APP β-CTF). A rectangle in each construct represents a signaling peptide. J. Immunoblot analysis showing comparable expression levels of deletion mutants.

Arrowhead, β -CTF; Arrow, α -CTF. K–N. Cultured hippocampal neurons (DIV21) were co-transfected with EGFP and each deletion construct as indicated. O. Quantification of spine density (10 neurons/group, * p <0.05; ** p <0.01).

Figure 2.

Effect of APP on spine formation in developing neurons. A. Cultured hippocampal neurons (DIV14) were transfected with expression vectors encoding EGFP and each deletion construct of APP ($\Delta E1$, $\Delta E1E2$, and β -CTF). B. Quantitative analysis of spine density (10 neurons/group, * $p < 0.05$; ** $p < 0.01$).

Figure 3.

APP affects clustering of synaptic proteins. A–F. Cultured hippocampal neurons (DIV21) were transfected with expression vectors encoding EGFP and empty vector, APP, APP M671V, or shRNA against APP as indicated. To rescue the RNAi effect, a shRNA-resistant form of APP (human APP) was also expressed with APP shRNA. Postsynaptic marker PSD-95 was immunostained 3 days after transfection. G–H. Quantification of puncta density of PSD-95 (10 neurons/group, $*p<0.05$; $**p<0.01$). I–N. Hippocampal neurons (DIV21) were transfected with expression vectors encoding EGFP and empty vector, APP, APP-M671V, APP-shRNA, APP-shRNA plus rescue construct as indicated. 3 days after transfection, neurons were immunostained against presynaptic marker Basson. O–P. Quantification of puncta density of Basson (10 neurons/group, $*p<0.05$; $**p<0.01$).

Figure 4.

APP regulates the expression of GluR2 subunits of AMPA receptors. A–E. Cultured hippocampal neurons (DIV21) were transfected with expression vectors encoding EGFP and empty vector, APP, APP-shRNA, or APP-shRNA plus rescue construct as indicated. Immunocytochemistry of surface GluR2 (sGluR2) was conducted under non-permeablized conditions. F–G. Quantification of integrated intensity of surface GluR2 (10 neurons/group, $*p<0.05$). H–L. Cultured neurons were transfected with expression vectors encoding EGFP and empty vector, APP, APP-shRNA, or APP-shRNA plus rescue construct as indicated. Immunocytochemistry of total GluR2 (tGluR2) was conducted under permeablized conditions. M and N. Quantification of integrated intensity of total GluR2 (10 neurons/group, $*p<0.05$; $**p<0.01$). O–R and T–W. Cultured neurons (DIV14) were transfected with expression vectors encoding EGFP and individual deletion constructs of APP ($\Delta E1$, $\Delta E1E2$, or β -CTF). Surface and total GluR2 (sGluR2 and tGluR2, respectively) were immunostained as indicated. S and X. Quantification of integrated intensity of surface and total GluR2 (10 neurons/group, $*p<0.05$; $**p<0.01$; $***p<0.001$).

Figure 5.

Effect of APP on the expression of GluR1. A–E. Cultured hippocampal neurons (DIV21) were transfected with expression vectors encoding EGFP and empty vector, APP, APP-shRNA, or APP-shRNA plus rescue construct as indicated. Immunocytochemistry of surface GluR1 was conducted under non-permeablized condition. F–G. Quantification of integrated intensity of surface GluR1 (10 neurons/group). H–L. Cultured neurons were transfected with expression vectors encoding EGFP and empty vector, APP, APP-shRNA, or APP-shRNA plus rescue construct as indicated. Immunocytochemistry of total GluR1 was conducted under permeablized condition. M–N. Quantification of integrated intensity of total GluR1 (10 neurons/group).

Figure 6.

Spine density and length in APP-deficient mice. A, Representative micrograph of a hippocampal CA1 neuron illustrating dendritic regions analyzed. B, Representative dendritic segments of CA1 pyramidal neurons from 12-month-old wild-type (WT), heterozygote (HT), and APP knock-out (KO) animals (N=6 mice/genotype). Scale bar, 5 μ m. C, Mean spine density from WT and APP KO mice in CA1 regions (28 neurons/genotype, * p <0.05; ** p <0.01). D, Cumulative distribution plot of hippocampal spine length from WT and APP-deficient mice (230–250 spines/genotype, Kolmogorov-Smirnov Test, p <0.001). E, Representative dendritic segments of cortical layer II/III pyramidal neurons from 12-month-old wild-type (WT), heterozygote (HT), and APP knock-out (KO) animals (N=6 mice/genotype). Scale bar, 5 μ m. CTX, cortex. F, Mean spine density from WT, HT, and KO mice (28 neurons/genotype, * p <0.05; ** p <0.01, *** p <0.001). G, Cumulative distribution plot of cortical layer II/III pyramidal neurons spine length from WT and APP-deficient mice (230–250 spines/genotype, Kolmogorov-Smirnov Test, p <0.001).

Figure 7.

Spine density and length in APP-transgenic mice. A. Representative dendritic segments of hippocampal CA1 region (left) and cortical layer II/III pyramidal neurons (right) from 12-month-old WT and APP transgenic (TG) mice (N=4 mice/genotype). Scale bars, 5 μm B. Mean spine density from WT and APP TG animals in hippocampal CA1 region (28 neurons/genotype, * $p<0.05$). C. Cumulative distribution plot of hippocampal CA1 region spine length in 12 month old WT and APP- transgenic mice (230–250 spines/genotype, Kolmogorov-Smirnov Test, $p<0.001$). D. Mean spine density from 12 month old WT and APP TG animals in cortical layer II–III pyramidal neurons (28 neurons/genotype, * $p<0.05$). E. Cumulative distribution plot of cortical layer II/III III pyramidal neurons spine length in 12 month old WT and APP- transgenic mice (230–250 spines/genotype, Kolmogorov-Smirnov Test, $p<0.001$). F. Representative dendritic segments of hippocampal CA1 region (left) and cortical layer II/III pyramidal neurons (right) from 1-month-old WT and APP

transgenic (TG) mice (N=4 mice/genotype). Scale bars, 5 μ m. G. Mean spine density in hippocampal CA1 pyramidal neurons from 1 month old WT and APP-transgenic mice (25 neurons/genotype, $**p < 0.01$). H. Cumulative distribution plot of hippocampal CA1 region spine length in 1 month old WT and APP-transgenic mice (230–250 spines/genotype, Kolmogorov-Smirnov Test, $p < 0.001$). I. Mean spine density from 1 month old WT and APP TG animals in cortical layer II–III pyramidal neurons (28 neurons/genotype, $*p < 0.05$). J. Cumulative distribution plot of cortical layer II/III pyramidal neurons spine length in 1 month old WT and APP-transgenic mice (230–250 spines/genotype, Kolmogorov-Smirnov Test, $p < 0.001$).

Figure 8.

NMR analysis of amino acid transmitters in APP knockout and transgenic mice brains. A and D, Metabolic flux of ^{13}C label into individual isotopomers of metabolites in whole brain tissue extracted in 6% perchloric acid (Mann Whitney test, $n=4/\text{genotype}$, $*p<0.05$). Glu, glutamate; Gln, glutamine; Lac, lactate; Asp, aspartate; GABA, gamma-amino butyric acid. B and E, Percentage of incorporation of ^{13}C into isotopomers of glutamate, lactate, GABA, glutamine and aspartate detected by $[^1\text{H-decoupled}]^{13}\text{C}$ NMR. C and F, Total amount of metabolites in whole brain tissue as shown by $[^{13}\text{C-decoupled}]^1\text{H}$ NMR.