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Deletion of the amyloid precursor-like protein 2 (APLP2) does not affect hippocampal neuron morphology or function

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Abstract

Amyloid precursor protein (APP), the parent molecule to amyloid β peptide, is part of larger gene family with two mammalian homologues, amyloid precursor-like protein 1 (APLP1) and amyloid precursor-like protein 2 (APLP2). Initial knock-out studies demonstrated that while single APP family gene deletions produced relatively mild phenotypes, deficiency of APLP2 and one other member of the gene family resulted in perinatal lethality, suggesting vital roles masked by functional redundancy of the other homologues. Because of the importance of APP in Alzheimer's disease, the vast majority of studies to date have concentrated on the neuronal functions of APP, leaving limited data on its homologues. APLP2 is of particular interest as it contains high sequence homology with APP, is processed similarly, is expressed in overlapping spatial and temporal patterns, and is obligatory for lethality when combined with deficiency of either APLP1 or APP but does not contain the toxic amyloid β sequence. Here we sought to test the role of APLP2 on neuronal structure and function using a combined approach involving *in vitro* and *in vivo* techniques in young and aged animals. Surprisingly, we found that unlike APP, APLP2 appears not to be essential for maintenance of dendritic structure, spiny density, or synaptic function. Thus, there is clear divergence in the functional redundancy between APP and APLP2.

Keywords

amyloid precursor-like protein 2; amyloid precursor protein; Alzheimer's disease; synaptic plasticity; dendritic spine; synapse

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Competing interests

The authors declare no competing interests.

Background

Alzheimer's disease (AD) is a neurodegenerative disorder of the central nervous system (CNS) and the most common form of dementia in the elderly population. In addition to the presence of extracellular senile plaques and intraneuronal neurofibrillary tangles (NFTs), there is significant neuronal and synaptic loss in the neocortex. Although AD aetiology remains unclear, neuronal and synaptic degeneration are thought to underlie the cause for cognitive decline, which is recognized to arise, at least in part, from the neurotoxic effects of amyloid β peptides (A β), derived from the proteolytic cleavage of the amyloid precursor protein (APP) (Hardy and Selkoe, 2002).

APP belongs to a family of proteolysis-dependent type-1 glycoproteins that includes the mammalian proteins amyloid precursor-like protein 1 (APLP1) and amyloid precursor-like protein 2 (APLP2) (Coulson et al., 2000). These transmembrane proteins exhibit high sequence homology and undergo similar processing by multiple secretases, resulting in a number of secreted fragments (Eggert et al., 2004), though neither APLP1 nor APLP2 contains the A β sequence. Orthologues of the APP family are well conserved and documented in *C. elegans*, *D. melanogaster*, *X. laevis*, *D. rerio*, and rodents (Hornsten et al., 2007; Lee and Cole, 2007; Luo et al., 1992; van den Hurk et al., 2001). APP and APLP2, but not APLP1, are present in *X. laevis*, suggesting that APLP1 is phylogenetically more recent (Collin et al., 2004). Rodent studies revealed that deletion of a single gene from this family produces mice that are viable and fertile with relatively mild phenotypes (von Koch et al., 1997; Zheng et al., 1995), while two of three double knockouts (APP^{-/-};APLP2^{-/-} and APLP2^{-/-};APLP1^{-/-}) and the triple knockout lead to perinatal lethality (Heber et al., 2000; Herms et al., 2004; von Koch et al., 1997; Zheng et al., 1995). This connotes a high degree of functional redundancy between the homologues and the fact that the APLP1^{-/-};APP^{-/-} are still viable and fertile with a relatively mild phenotype distinguishes APLP2 as containing a unique and vital function. Although this redundancy has led to difficulties in identifying and interpreting defined roles, further studies of APP indicate discrete functions in numerous neuronal processes such as: cell adhesion, dendritic outgrowth, axonal transport, synapse formation and synapse modulation and this diversity of roles is attributed to its numerous proteolytic products (reviewed in (Jacobsen and Iverfeldt, 2009)).

Detailed analyses of the mammalian homologues and their metabolites have been far less extensive, although these studies may prove to be vital for understanding the pathogenesis of AD. It is known that APLP2 and APP contain the highest degree of sequence homology within this family of genes and that they are expressed ubiquitously and in an overlapping spatial and temporal pattern (Wasco et al., 1993). Additionally, the double deletion of APP and APLP2 culminates in perinatal lethality and abnormal synapse formation and function at the neuromuscular junction (von Koch et al., 1997; Wang et al., 2005). Our overall goal is to define the respective roles of APP and its homologues and in this study, we specifically focused on whether the loss of APLP2 leads to neuronal abnormalities by quantitatively analyzing dendritic arborization, spine density, spine morphology and neuronal function. Unlike our recent results demonstrating multiple defects in neuronal structure and function in APP deficient mice, loss of APLP2 resulted in no detectable abnormalities in these same parameters.

Results

APLP2 and spine density in primary hippocampal neurons

Initial studies of APLP2^{-/-} mice reported that these animals were viable and fertile, with no gross phenotype (von Koch et al., 1997). However, this report was limited and no subsequent studies have analyzed finer neurological measurements, traits that have been

attributed to APP. Recently, our lab found that primary hippocampal neurons from APP^{-/-} mice showed marked reduction in dendritic spines (~35%) when compared with their wt counterparts (Tyan, 2010). This result is striking when compared to the subtle phenotypes previously reported in the APP^{-/-} animals and this prompted us to examine whether the deletion of APLP2 would affect spine density similarly. For this experiment, primary neuronal cultures were established from the hippocampus of APLP2^{-/-} and wt littermates and the dendritic spines were quantified after neurons were transfected with eGFP at 15–18 DIV. Unlike APP deficient neurons, primary hippocampal neurons cultured from APLP2^{-/-} mice contained the same number of spines as compared to neurons cultured from the hippocampi of wt littermates (Fig. 1; APLP2^{+/+}: 7.1 ± 0.13 spines/10 μm; APLP2^{-/-}: 6.76 ± 0.1 spines/10 μm, p > 0.05).

APLP2 and spine density in vivo

Although the accruing data attest to the notion that APP may be important for synapse formation/maintenance *in vivo*, there is still very little data on whether this is a shared function of APLP2. The results from cultured neurons indicated that loss of APLP2 did not affect the number of dendritic spines in an *in vitro* setting. Several of the neuronal abnormalities displayed by the APP^{-/-} mice appear to be age-dependent with the majority of deficits evident by 8–12 months of age but not at 1–4 months of age (Dawson et al., 1999; Phinney et al., 1999; Senechal et al., 2008; Zheng et al., 1995), including synaptic density. Accordingly, we investigated whether the loss of APLP2 affects spine and dendritic morphology in CA1 neurons *in vivo* in aged mice, defined here as 10–12 month old animals. Digitally reconstructed dendritic segments from APLP2^{-/-} and wt mice are depicted in Figure 2A. Consistent with our data from primary cultures, we found that the absence of APLP2 did not affect spine density in aged mice (APLP2^{+/+} = 2.33 ± 0.14 spines/μm; APLP2^{-/-} = 2.15 ± 0.09 spines/μm, p > .05) (Fig. 2B). Next, spines were automatically and systematically measured and categorized into three subtypes: stubby, thin, or mushroom. Each spine subtype contains distinct properties and the proportion of spine subtypes is thought to reflect biological differences or changes in sensory input (Alvarez and Sabatini, 2007). Here, we found that the percentage of spine subtypes were comparable across genotypes (Stubby: APLP2^{+/+} = 35.5 ± 1.4%; APLP2^{-/-} = 37.2 ± 1.2%; Thin: APLP2^{+/+} = 48.2 ± 1.7%; APLP2^{-/-} = 47.8 ± 1.5%; Mushroom: APLP2^{+/+} = 16.3 ± .8%; APLP2^{-/-} = 15.0 ± 1.4%, p > .05 for all comparisons) (Fig. 2C). Furthermore, we measured and compared spine volume and found that loss of APLP2 did not affect total mean spine volume, nor does it affect mean spine volume per spine subtype (Stubby: APLP2^{+/+} = 0.05 ± 0.002 μm³; APLP2^{-/-} = 0.05 ± 0.002 μm³; Thin: APLP2^{+/+} = 0.037 ± 0.001 μm³; APLP2^{-/-} = 0.037 ± 0.001 μm³; Mushroom: APLP2^{+/+} = 0.145 ± 0.007 μm³; APLP2^{-/-} = 0.142 ± 0.009 μm³, p > .05 for all comparisons) (Fig. 2D).

Dendritic length and arborization of APLP2^{-/-} mice

Previous reports showed that the CA1 pyramidal neurons from APP^{-/-} mice displayed a reduced maximum projection length and a reduction in total dendritic length at 8–12 months of age (Seabrook et al., 1999) but our own recent studies demonstrated that this decrease in total dendrite length and reduced dendritic arborization was age dependent as these alterations were absent in 4-month old mice (Tyan, 2010). Primary hippocampal cultures from APLP2^{-/-} mice revealed no obvious change in dendrite formation or arborization, however, whether this is true *in vivo* and in aged animals are not known. To study this, the dendritic tree of CA1 pyramidal neurons were imaged and digitally reconstructed. Representative examples of CA1 dendritic arbor reconstructions and dendrograms are depicted in Figure 3A–B. As for the spine density measurements above, total dendritic length was not altered in the APLP2^{-/-} mice when compared to controls (APLP2^{+/+} = 1780 ± 118 μm; APLP2^{-/-} = 1878 ± 131 μm, p > .05) (Fig. 3C). Additionally, detailed analyses of

the various morphometric parameters (i.e., length and complexity) of individual apical dendrites in APLP2^{-/-} mice and wt controls showed no significant differences in dendrite length or number of intersections per radial region from the soma ($p > .05$ for all measurements) (Fig. 3D–E).

Deletion of APLP2 and synaptic function

Static measurements of neuronal structure may not accurately reflect actual synaptic function. Further, it has been reported that changes in synaptic function might also be age dependent in APP deficient mice (Dawson et al., 1999; Seabrook et al., 1999; Senechal et al., 2008; Tyan, 2010), thus we tested synaptic basal synaptic transmission and synaptic plasticity in both young and aged APLP2 deficient animals. First, extracellularly recorded field excitatory post synaptic potentials (fEPSPs) were used to assess the strength of basal synaptic transmission at the Schaffer collateral to CA1 synapse in acute hippocampal slices in young (1–2 months) and aged (10–12 months) mice as compared to their wt littermates. The input/output ratios were calculated and we found that the ratio was comparable between genotypes and across both age groups (Young: APLP2^{+/+} = 4.35 ± 0.20 ; APLP2^{-/-} = 4.27 ± 0.21 , $p > .05$; Aged: APLP2^{+/+} = 4.23 ± 0.28 ; APLP2^{-/-} = 4.43 ± 0.3 , $p > .05$) (Fig. 4A). Simultaneously, we measured paired-pulse facilitation (PPF), which is defined as an increase in the fEPSP when an electrical pulse is elicited shortly after a first. A change in PPF reflects a change in the availability of synaptic vesicles for release and upon comparison of the paired-pulse ratio (fEPSP₂/fEPSP₁), we found that PPF remains unaffected by the absence of APLP2 (Young: APLP2^{+/+} = 1.59 ± 0.09 ; APLP2^{-/-} = 1.57 ± 0.03 , $p > .05$; Aged: APLP2^{+/+} = 1.46 ± 0.05 ; APLP2^{-/-} = 1.37 ± 0.03 , $p > .05$) (Fig. 4B). Finally, we tested for impairments in synaptic plasticity by measuring long-term potentiation (LTP), the cellular process thought to underlie learning and memory (Kessels and Malinow, 2009). To induce LTP, four 1-s tetanic stimuli were given at 100 Hz, 5 minutes apart and fEPSPs were monitored for 60 minutes. We report that the percent increase in fEPSPs post-tetanus was indistinguishable between genotypes at each age group (Young: APLP2^{+/+} = $191 \pm 7\%$; APLP2^{-/-} = $195 \pm 7\%$, $p > .05$; Aged: APLP2^{+/+} = $197 \pm 13\%$; APLP2^{-/-} = $180 \pm 17\%$, $p > .05$) (Fig. 5).

Discussion

APLP2 contains a high degree of sequence and structural homology to APP but does not contain the A β region. This, coupled with an almost identical spatial and temporal pattern of expression presents a unique opportunity to explore whether the non-A β metabolites play a role in AD pathogenesis. Although initial reports on APLP2^{-/-} mice indicated no overt phenotype (von Koch et al., 1997), these studies are few and until recently, none had investigated in detail neuronal morphology, synaptic function, or spine formation, the physiological roles that have been attributed to APP. Here, we showed that APLP2 did not appear to be essential for spine numbers in primary hippocampal cultures. Our results further indicated that APLP2 did not seem to determine or maintain spine density, type, or volume in CA1 pyramidal neurons *in vivo* in animals of 10–12 months of age. APLP2 by itself was also not required for proper dendrite elongation and organization in aged mice as assessed by total dendrite length, dendritic length per radial distance from the soma and number of intersections of dendrites per radial distance from the soma. Finally, we demonstrated that APLP2^{-/-} mice did not exhibit any impairment in neuronal function as measured by basal synaptic transmission, paired-pulse ratio, and long-term potentiation at the CA1–CA3 synapses in young or old animals.

Although the initial report showed no overt upregulation of APP or APLP1 in APLP2^{-/-} mice (von Koch et al., 1997), animal model studies leave little debate for the existence of functional redundancy. Although we cannot exclude a physiological role of APLP2 in

synapse formation and function *in vivo*, the fact that endogenous APLP2 cannot functionally compensate for loss of APP suggests that APP contains neuronal functions that are either distinct from APLP2 or dominant to APLP2. A similar situation is at play in the two members of the presenilin gene family *PSEN1* (PS1) and *PSEN2* (PS2). Loss of PS1 is embryonically lethal (Shen et al., 1997; Wong et al., 1997), yet loss of PS2 leads to viable animals with only mild phenotype (Herreman et al., 1999). Nonetheless, both PS1 and PS2 perform overlapping functions as familial AD mutations in both genes invariably result in alterations in the ratio of A β peptides, favoring the generation of the longer A β 42 isoforms (Tanzi and Bertram, 2005).

Although a potentially useful tool, knockouts of APP or APLP1 coupled with APLP2 deficiency leads to perinatal lethality in 80% of pups (Heber et al., 2000; von Koch et al., 1997). In pursuit of distinguishing the domain(s) responsible for phenotype, it was recently reported that a substitution of tyrosine at position 682 to glycine in the YENPTY motif in the APP cytoplasmic domain was sufficient to induce this postnatal lethality when crossed against the APLP2^{-/-} background, indicating that the C-terminal domain is essential for APP function (Barbagallo et al., 2011). Supporting this notion, mice with knock-in of sAPP β into the APLP2^{-/-} background, thus without the transmembrane or cytoplasmic domains of APP, still exhibited lethality and neuromuscular defects (Li et al., 2010). However, knock-in of sAPP α into similar APLP2 null background rescued the postnatal lethality although surviving mice showed neuromuscular abnormalities and impaired learning and memory, indicating that these are hypomorphic with respect to APP function (Weyer et al., 2011). Taken together, these studies indicated that functional domains are present in both the N-terminal ectodomain and the C-terminal cytoplasmic. Whether similar functional domains are present in APLP2 is not known. However, because APLP2 does not have the A β sequence, thus lacking the 17 amino acids at the C-terminus of sAPP α , one would predict that APLP2 does not have a similar trophic region in the juxtamembrane region. Notably, it was also shown that LTP appeared normal in APLP2 deficient mice, results entirely consistent with what we observed. Given our results, we conclude that like the electrophysiological properties, the neuronal morphology of APLP2 deficient animals is also normal.

The majority of studies indicate that genetic ablation of APP leads to a loss of function as implicated by a reduction in spines, reduced dendritic complexity and impaired synaptic function (Fitzjohn et al., 2000; Seabrook et al., 1999; Zheng et al., 1995). However, a few studies reported contradicting results, such as increased synapse number, miniature EPSP frequency and AMPA and NMDA currents (Bittner et al., 2009; Priller et al., 2006). The reasons for these discrepancies are unknown but in addition to different experimental approaches and mouse strains, the age and brain region that were studied may explain some of these results. For example, our own studies recently demonstrated a decrease in spine density and reduced dendritic length and complexity in CA1 pyramidal cells at 12–14 months but not at 4 months of age (Tyan, 2010). This surprising age-dependent change in neuronal morphology was not described in previous studies that examined only older animals. Given these results, we hypothesized that APLP2 deficiency may also display an age dependent phenotype. However, our electrophysiological experiments did not show any defects in either young or aged animals, thereby demonstrating convincingly that there is no age dependent alteration in synaptic function in APLP2 deficient animals.

Regardless, we currently favor the hypothesis that the APP family members share similar functions through the conserved domains. In part, we reason that while little work has been done on the neurotrophism of APLP2, there is evidence that it has neurotrophic activities (Cappai et al., 1999; Young-Pearse et al., 2008) and the overexpression of APP, APLP2, or APLP1, was each shown to rescue the migration defect seen in neuronal precursor cells

derived from triple knockout animals (Young-Pearse et al., 2007). Similarly, it has been reported that sAPP β and the cognate APLP2 region contain a similar function in axonal pruning (Nikolaev et al., 2009), reemphasizing a broad spectrum of functional redundancy.

In conclusion, we found that unlike APP, APLP2 is dispensable for normal growth and maintenance of dendritic growth and arborization, spine formation, and neuronal function in an age-independent manner. Although it is interesting to surmise the source of change in function between APP and APLP2, it is likely that functional redundancy blurs the physiologic functions of each of these proteins. Future experiments may need to be carried out using a temporally controlled expression system in order to limit these compensatory effects and to help resolve the complex interplay amongst this family of genes.

Methods

Animals

APLP2^{-/-} mice were kindly provided by Dr. Hui Zheng at Baylor College of Medicine and generated as described in von Koch et al. (1997) (von Koch et al., 1997). Briefly, the APLP2 gene was inactivated by excision of the promoter and exon 1, thereby prohibiting transcription and translation of the APLP2 protein. The transgenic line was maintained by crossing with C57BL/6 breeders (Jackson Laboratories). APLP2^{-/-} and wt (APLP2^{+/+}) mice were generated from heterozygous pairings. All animal procedures were approved by the Institutional Animal Care and Use Committee at University of California, San Diego and in accordance with the National Institutes of Health guidelines.

Primary hippocampal culture

Hippocampal cultures were prepared and transfected as described elsewhere (Calabrese et al., 2007). Hippocampi were excised from APLP2^{-/-} mice and control littermates on postnatal days 0–1 (P0–P1), trypsinized and plated at a density of 300 cells/mm² on poly-L-lysine (Sigma, St. Louis, MO, USA) coated coverslips. Cultures were maintained at 37°C and 5% CO₂ in neurobasal medium supplemented with 2% B-27, 2 mM L-glutamine and 25 μ g/ml sodium pyruvate and the medium was changed every 3–4 days (unless noted, reagents from Invitrogen, Carlsbad, CA, USA). At 14–18 days *in vitro* (DIV) neurons were transfected with enhanced green fluorescent protein (eGFP) using a low efficiency calcium phosphate precipitation protocol. Briefly, medium from the neuronal cultures was replaced with new neurobasal medium plus 2% B27 incubated at 37°C, 5% CO₂ for 30 minutes. A DNA, H₂O and CaCl₂ mixture was added to 2X HEPES Buffered Solution (HBS), pH 7.07, incubated for 20 minutes at room temperature (RT), added drop-wise to hippocampal cultures and incubated at 37°C, 5% CO₂ for approximately 3.5 hours, or until cultures displayed ample precipitate. Cultures were then carefully washed twice with 37°C 2X HBS, replacing only 50% of media per wash. Solution was then completely replaced with the pre-transfection conditioned neurobasal medium and fixed 1 day post-transfection.

Image acquisition and spine quantification in primary hippocampal cultures

Photomicrographs were obtained using an Olympus DSU IX-81 inverted microscope fitted with a spinning disk confocal attachment and a 60x/1.2 N.A. water immersion objective. Labeled transfected neurons were chosen randomly for imaging from neuronal cultures from three coverslips. For all dendritic spine analyses, the region of the apical dendrites after the first branch point was selected (secondary dendrite). Dendritic spine density was scored from three randomly chosen areas per neuron. Z-sections were taken at 0.3- μ m intervals and were stacked with maximum projection. All analyses were conducted blind to genotype. Data were expressed as means $\pm$ SEM from independent replicates.

Primary hippocampal cultures were fixed on coverslips by adding a pre-warmed mixture of 4% sucrose/4% paraformaldehyde (Sigma, St. Louis, MO, USA) in phosphate-buffered saline (PBS) for 15 minutes at 37 °C. This was replaced with fresh 4% sucrose/4% paraformaldehyde in PBS for 15 minutes at RT. Cultures were then rinsed (3X with PBS) and permeabilized with 0.3% Triton X-100 for 10 minutes at RT and rinsed again. Neurons were blocked for 1 hour with 5% goat serum at 37 °C, rinsed with PBS. Coverslips were mounted with Prolong gold antifade reagent (Molecular Probes, Eugene, OR, USA).

Intracellular dye injections

Mice were anesthetized with chloral hydrate (0.1 ml of a 15% solution, i.p.), and transcardially perfused with ice-cold 1% paraformaldehyde in 0.1 M PBS (pH 7.4) for one minute. This was followed by perfusion of 4% paraformaldehyde in 0.1 M PBS with 0.125% glutaraldehyde for 12 minutes. The brains were removed from the skull and postfixed overnight at 4°C in 4% paraformaldehyde in PBS with 0.125% glutaraldehyde and then transferred to PBS and sectioned on a Vibratome (Leica VT1000S, Bannockburn, IL, USA) into 200 µm sections and stored at 4°C in PBS.

For intracellular injections, sections were incubated in 4',6-diamidino-2-phenylindole (DAPI; Sigma) for 5 minutes to reveal the cytoarchitectural features of the pyramidal layer of CA1 of the hippocampus. The sections were then mounted on nitrocellulose paper and immersed in ice-cold 0.1 M PBS. Pyramidal neurons in the CA1 were injected with an intracellular iontophoretic injection of 5% Lucifer Yellow (Molecular Probes, Eugene, OR, USA) in distilled water under a DC current of 3–8 nA for 5–10 minutes, or until dye had completely filled distal processes and no further loading was observed (Duan et al., 2002; Duan et al., 2003; Hao et al., 2006; Radley et al., 2006; Radley et al., 2004). Five to 10 neurons were injected per slice and placed far enough apart to avoid overlapping of their dendritic trees. Brain sections containing loaded neurons were then mounted on gelatin-coated glass slides and cover slipped in PermaFluor mounting medium (Immunotech).

Neuronal and dendritic reconstruction

In order for a loaded neuron to be included in the analysis, it had to satisfy the following criteria: (1) lie within the pyramidal layer of the CA1 as defined by cytoarchitectural characteristics; (2) demonstrate complete filling of dendritic tree, as evidenced by well-defined endings; (3) demonstrate intact primary and secondary branches; (4) demonstrate intact tertiary branches, with the exception of branches that extended beyond 50 µm in radial distance from the cell soma (Duan et al., 2002; Duan et al., 2003; Hao et al., 2006; Liston et al., 2006; Radley et al., 2004). Neurons meeting these criteria were reconstructed in 3-dimension (3D) with a 63x/1.4 N.A., Plan-Apochromat oil immersion objective on a Zeiss Axiophot 2 microscope equipped with a motorized stage, video camera system, and Neurolucida morphometry software (MBF Bioscience, Williston, VT, USA). Using NeuroExplorer software (MBF Bioscience) total dendritic length, number of intersections and the amount of dendritic material per radial distance from the soma, in 30-µm increments (Sholl, 1953) were analyzed in order to assess morphological cellular diversity and potential differences among animal groups.

Confocal microscopy and *in vivo* spine acquisition

Using an approach that precludes sampling bias of spines, dendritic segments were selected with a systematic-random design (Hao et al., 2006; Radley et al., 2006). Dendritic segments, 20–25 µm in length, were imaged on the a Zeiss LSM 510 confocal microscope (Zeiss, Thornwood, NY, USA) using a 100X/1.4 N.A. Plan-Apochromat objective with a digital zoom of 3.5 and an Ar/Kr laser at an excitation wavelength of 488 nm. All confocal stacks were acquired at 512×512 pixel resolution with a z-step of 0.1 µm, a pinhole setting

of 1 Airy unit and optimal settings for gain and offset. All confocal stacks included approximately 1 μm above and below the identified dendritic segment. On average 3 z-stacks were imaged per neuron. In order for a dendritic segment to be optically imaged it had to satisfy the following criteria: (1) the entire segment had to fall within a depth of 50 μm ; (2) dendritic segments had to be either parallel or at acute angles to the coronal surface of the section; and (3) segments did not overlap other segments that would obscure visualization of spines (Radley et al., 2006; Radley et al., 2008). Confocal stacks were then deconvolved using an iterative blind deconvolution algorithm (AutoDeblur version 8.0.2; MediaCybernetics, Bethesda, MD, USA). This step is necessary since in the raw image, the image spread in the Z plane would limit the precise interpretation of 3D spine morphology.

Spine analysis

After deconvolution, confocal stacks were analyzed with NeuronStudio (Rodriguez et al., 2008; Rodriguez et al., 2006; Wearne et al., 2005) (<http://www.mssm.edu/cnic>) to examine global and local morphometric characteristics of dendrites and spines such as dendritic spine densities, dendritic spine shape (stubby, thin and mushroom), and dendritic spine volume. This software allows for automated digitization and reconstructions of 3D neuronal morphology from multiple confocal stacks on a spatial scale and averts the subjective errors encountered during manual tracing using a Rayburst-based spine analysis (Fitzjohn et al., 2000; Rodriguez et al., 2003; Rodriguez et al., 2008; Rodriguez et al., 2006). At least 5 neurons per mouse and at least 3 apical and 3 basal dendritic segments per neuron were analyzed with each segment manually inspected and appropriate corrections made using the NeuronStudio interface.

Acute Slice Preparation

Animals were anesthetized with isoflurane, decapitated, and brains were rapidly removed and placed into oxygenated ice-cold sucrose cutting buffer (mM: 83 NaCl, 2.5 NaHCO₃, 3.3 MgSO₄, 0.5 CaCl₂, 2.5 KCl, 80 sucrose and 22 glucose). A vibrating blade microtome (Leica) was used to prepare 400 μm -thick whole brain slices which were immediately placed into a recovery chamber containing oxygenated artificial cerebral spinal fluid (ACSF) (mM: 118 NaCl, 1.2 NaH₂PO₄, 3.7 KCl, 2.6 NaHCO₃, and 11 glucose) which was incubated at 37°C for 45 minutes and then left at RT for an additional 45 minutes. Before analysis, the slice was carefully transferred to a submerged recording chamber and perfused with aCSF at 2 ml/minute and maintained at 29°C.

Extracellular Field Recording

Extracellularly recorded field excitatory postsynaptic potentials (fEPSPs) were recorded from in the striatum radiatum of the CA1 region of the hippocampus of mice at 1–2 months and 10–12 months of age. fEPSPs were evoked using a bipolar electrode (FHC, Bowdoin, ME, USA) positioned in the Schaffer collateral/commissural pathway. Stimuli were generated with a GRASS S88 stimulator coupled to an Iso-flex stimulation isolation unit (AMPI, Jerusalem, Israel) and recorded using glass recording pipette filled with oxygenated ACSF solution (tip resistance, 1.5–2.5 M Ω) coupled to an Axopatch 1D amplifier (Molecular Devices, Sunnyvale, CA, USA). Data were analyzed using Igor (WaveMetrics Incorporated, Tigard, OR, USA). Basal synaptic transmission was assessed by comparing the input and output relationship of the fEPSPs recorded. The input–output relationship (I/O) was calculated by dividing the peak amplitude of the fEPSP by the peak amplitude of the fiber volley. Pre-synaptic release was examined using paired pulse facilitation. A second pulse was elicited 50 ms after the initial stimulation and ratio was calculated by dividing the peak amplitude of the second pulse by the peak amplitude of the first pulse. Long-term potentiation (LTP) was induced by four high frequency stimulations (HFS) delivered 5 minutes apart, each at 100 Hz for 1 s after a 20-minute baseline period. Baseline was set at

20–30% the maximum field response and stimulation was maintained at a frequency of .033 Hz. fEPSPs were monitored for 60 minutes after the final tetanus (Townsend et al., 2006). Each five consecutive data points were then pooled and averaged. For final analyses, each of these pooled data points was averaged across experiments for the final 30 minutes of each experiment and compared across samples.

Statistical analysis

Scores for analyses for each APLP2^{-/-} animal and their wt counterparts were obtained by taking the average of the data from all neurons for each animal. An independent samples *t* test was performed on spine density, spine percentage, spine volume, dendritic length, and electrophysiology experiments. Sholl analysis data were compared by repeated measures ANOVA with genotype (APLP2^{-/-} vs. APLP2^{+/+}) as the between-group factor and distance from the soma as the repeated-measures factor. The α level was set at 0.05 for all analyses in the study, and all data were represented as mean $\pm$ SEM.

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Abbreviations

AD	Alzheimer's disease
Aβ	amyloid-beta peptide
APP	amyloid precursor protein
APLP1	amyloid precursor-like protein 1
APLP2	amyloid precursor-like protein 2
DIV	days <i>in vitro</i>
eGFP	enhanced green fluorescent protein
DAPI	4'6-diamidino-2-phenylindole
fEPSP	field excitatory post synaptic potential
LTP	Long-term potentiation
HFS	high frequency stimulus
ACSF	artificial cerebral spinal fluid

References

- Alvarez VA, Sabatini BL. Anatomical and physiological plasticity of dendritic spines. *Annu Rev Neurosci.* 2007; 30:79–97. [PubMed: 17280523]
- Barbagallo AP, Wang Z, Zheng H, D'Adamio L. A single tyrosine residue in the amyloid precursor protein intracellular domain is essential for developmental function. *J Biol Chem.* 2011; 286:8717–8721. [PubMed: 21266574]

- Bittner T, Fuhrmann M, Burgold S, Jung CK, Volbracht C, Steiner H, Mitteregger G, Kretzschmar HA, Haass C, Herms J. Gamma-secretase inhibition reduces spine density in vivo via an amyloid precursor protein-dependent pathway. *J Neurosci*. 2009; 29:10405–10409. [PubMed: 19692615]
- Calabrese B, Shaked GM, Tabarean IV, Braga J, Koo EH, Halpain S. Rapid, concurrent alterations in pre- and postsynaptic structure induced by naturally-secreted amyloid-beta protein. *Mol Cell Neurosci*. 2007; 35:183–193. [PubMed: 17368908]
- Cappai R, Mok SS, Galatis D, Tucker DF, Henry A, Beyreuther K, Small DH, Masters CL. Recombinant human amyloid precursor-like protein 2 (APLP2) expressed in the yeast *Pichia pastoris* can stimulate neurite outgrowth. *FEBS Lett*. 1999; 442:95–98. [PubMed: 9923612]
- Collin RW, van Strien D, Leunissen JA, Martens GJ. Identification and expression of the first nonmammalian amyloid-beta precursor-like protein APLP2 in the amphibian *Xenopus laevis*. *Eur J Biochem*. 2004; 271:1906–1912. [PubMed: 15128300]
- Coulson EJ, Paliga K, Beyreuther K, Masters CL. What the evolution of the amyloid protein precursor supergene family tells us about its function. *Neurochem Int*. 2000; 36:175–184. [PubMed: 10676850]
- Dawson GR, Seabrook GR, Zheng H, Smith DW, Graham S, O'Dowd G, Bowery BJ, Boyce S, Trumbauer ME, Chen HY, Van der Ploeg LH, Sirinathsinghji DJ. Age-related cognitive deficits, impaired long-term potentiation and reduction in synaptic marker density in mice lacking the beta-amyloid precursor protein. *Neuroscience*. 1999; 90:1–13. [PubMed: 10188929]
- Duan H, Wearne SL, Morrison JH, Hof PR. Quantitative analysis of the dendritic morphology of corticocortical projection neurons in the macaque monkey association cortex. *Neuroscience*. 2002; 114:349–359. [PubMed: 12204204]
- Duan H, Wearne SL, Rocher AB, Macedo A, Morrison JH, Hof PR. Age-related dendritic and spine changes in corticocortically projecting neurons in macaque monkeys. *Cereb Cortex*. 2003; 13:950–961. [PubMed: 12902394]
- Eggert S, Paliga K, Soba P, Evin G, Masters CL, Weidemann A, Beyreuther K. The proteolytic processing of the amyloid precursor protein gene family members APLP-1 and APLP-2 involves alpha-, beta-, gamma-, and epsilon-like cleavages: modulation of APLP-1 processing by n-glycosylation. *J Biol Chem*. 2004; 279:18146–18156. [PubMed: 14970212]
- Fitzjohn SM, Morton RA, Kuenzi F, Davies CH, Seabrook GR, Collingridge GL. Similar levels of long-term potentiation in amyloid precursor protein -null and wild-type mice in the CA1 region of picrotoxin treated slices. *Neurosci Lett*. 2000; 288:9–12. [PubMed: 10869803]
- Hao J, Rapp PR, Leffler AE, Leffler SR, Janssen WG, Lou W, McKay H, Roberts JA, Wearne SL, Hof PR, Morrison JH. Estrogen alters spine number and morphology in prefrontal cortex of aged female rhesus monkeys. *J Neurosci*. 2006; 26:2571–2578. [PubMed: 16510735]
- Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science*. 2002; 297:353–356. [PubMed: 12130773]
- Heber S, Herms J, Gajic V, Hainfellner J, Aguzzi A, Rulicke T, von Kretzschmar H, von Koch C, Sisodia S, Tremml P, Lipp HP, Wolfer DP, Muller U. Mice with combined gene knock-outs reveal essential and partially redundant functions of amyloid precursor protein family members. *J Neurosci*. 2000; 20:7951–7963. [PubMed: 11050115]
- Herms J, Anliker B, Heber S, Ring S, Fuhrmann M, Kretzschmar H, Sisodia S, Muller U. Cortical dysplasia resembling human type 2 lissencephaly in mice lacking all three APP family members. *EMBO J*. 2004; 23:4106–4115. [PubMed: 15385965]
- Herreman A, Hartmann D, Annaert W, Saftig P, Craessaerts K, Serneels L, Umans L, Schrijvers V, Checler F, Vanderstichele H, Baekelandt V, Dressel R, Cupers P, Huylebroeck D, Zwijsen A, Van Leuven F, De Strooper B. Presenilin 2 deficiency causes a mild pulmonary phenotype and no changes in amyloid precursor protein processing but enhances the embryonic lethal phenotype of presenilin 1 deficiency. *Proc Natl Acad Sci U S A*. 1999; 96:11872–11877. [PubMed: 10518543]
- Hornsten A, Lieberthal J, Fadia S, Malins R, Ha L, Xu X, Daigle I, Markowitz M, O'Connor G, Plasterk R, Li C. APL-1, a *Caenorhabditis elegans* protein related to the human beta-amyloid precursor protein, is essential for viability. *Proc Natl Acad Sci U S A*. 2007; 104:1971–1976. [PubMed: 17267616]

- Jacobsen KT, Iverfeldt K. Amyloid precursor protein and its homologues: a family of proteolysis-dependent receptors. *Cell Mol Life Sci*. 2009; 66:2299–2318. [PubMed: 19333550]
- Kessels HW, Malinow R. Synaptic AMPA receptor plasticity and behavior. *Neuron*. 2009; 61:340–350. [PubMed: 19217372]
- Lee JA, Cole GJ. Generation of transgenic zebrafish expressing green fluorescent protein under control of zebrafish amyloid precursor protein gene regulatory elements. *Zebrafish*. 2007; 4:277–286. [PubMed: 18284334]
- Li H, Wang B, Wang Z, Guo Q, Tabuchi K, Hammer RE, Sudhof TC, Zheng H. Soluble amyloid precursor protein (APP) regulates transthyretin and Klotho gene expression without rescuing the essential function of APP. *Proc Natl Acad Sci U S A*. 2010; 107:17362–17367. [PubMed: 20855613]
- Liston C, Miller MM, Goldwater DS, Radley JJ, Rocher AB, Hof PR, Morrison JH, McEwen BS. Stress-induced alterations in prefrontal cortical dendritic morphology predict selective impairments in perceptual attentional set-shifting. *J Neurosci*. 2006; 26:7870–7874. [PubMed: 16870732]
- Luo L, Tully T, White K. Human amyloid precursor protein ameliorates behavioral deficit of flies deleted for *Appl* gene. *Neuron*. 1992; 9:595–605. [PubMed: 1389179]
- Nikolaev A, McLaughlin T, O'Leary DD, Tessier-Lavigne M. APP binds DR6 to trigger axon pruning and neuron death via distinct caspases. *Nature*. 2009; 457:981–989. [PubMed: 19225519]
- Phinney AL, Calhoun ME, Wolfer DP, Lipp HP, Zheng H, Jucker M. No hippocampal neuron or synaptic bouton loss in learning-impaired aged beta-amyloid precursor protein-null mice. *Neuroscience*. 1999; 90:1207–1216. [PubMed: 10338291]
- Priller C, Bauer T, Mitteregger G, Krebs B, Kretschmar HA, Herms J. Synapse formation and function is modulated by the amyloid precursor protein. *J Neurosci*. 2006; 26:7212–7221. [PubMed: 16822978]
- Radley JJ, Rocher AB, Miller M, Janssen WG, Liston C, Hof PR, McEwen BS, Morrison JH. Repeated stress induces dendritic spine loss in the rat medial prefrontal cortex. *Cereb Cortex*. 2006; 16:313–320. [PubMed: 15901656]
- Radley JJ, Rocher AB, Rodriguez A, Ehlenberger DB, Dammann M, McEwen BS, Morrison JH, Wearne SL, Hof PR. Repeated stress alters dendritic spine morphology in the rat medial prefrontal cortex. *J Comp Neurol*. 2008; 507:1141–1150. [PubMed: 18157834]
- Radley JJ, Sisti HM, Hao J, Rocher AB, McCall T, Hof PR, McEwen BS, Morrison JH. Chronic behavioral stress induces apical dendritic reorganization in pyramidal neurons of the medial prefrontal cortex. *Neuroscience*. 2004; 125:1–6. [PubMed: 15051139]
- Rodriguez A, Ehlenberger D, Kelliher K, Einstein M, Henderson SC, Morrison JH, Hof PR, Wearne SL. Automated reconstruction of three-dimensional neuronal morphology from laser scanning microscopy images. *Methods*. 2003; 30:94–105. [PubMed: 12695107]
- Rodriguez A, Ehlenberger DB, Dickstein DL, Hof PR, Wearne SL. Automated three-dimensional detection and shape classification of dendritic spines from fluorescence microscopy images. *PLoS One*. 2008; 3:e1997. [PubMed: 18431482]
- Rodriguez A, Ehlenberger DB, Hof PR, Wearne SL. Rayburst sampling, an algorithm for automated three-dimensional shape analysis from laser scanning microscopy images. *Nat Protoc*. 2006; 1:2152–2161. [PubMed: 17487207]
- Seabrook GR, Smith DW, Bowery BJ, Easter A, Reynolds T, Fitzjohn SM, Morton RA, Zheng H, Dawson GR, Sirinathsinghji DJ, Davies CH, Collingridge GL, Hill RG. Mechanisms contributing to the deficits in hippocampal synaptic plasticity in mice lacking amyloid precursor protein. *Neuropharmacology*. 1999; 38:349–359. [PubMed: 10219973]
- Senechal Y, Kelly PH, Dev KK. Amyloid precursor protein knockout mice show age-dependent deficits in passive avoidance learning. *Behav Brain Res*. 2008; 186:126–132. [PubMed: 17884188]
- Shen J, Bronson RT, Chen DF, Xia W, Selkoe DJ, Tonegawa S. Skeletal and CNS defects in Presenilin-1-deficient mice. *Cell*. 1997; 89:629–639. [PubMed: 9160754]
- Sholl DA. Dendritic organization in the neurons of the visual and motor cortices of the cat. *J Anat*. 1953; 87:387–406. [PubMed: 13117757]

- Tanzi RE, Bertram L. Twenty years of the Alzheimer's disease amyloid hypothesis: a genetic perspective. *Cell*. 2005; 120:545–555. [PubMed: 15734686]
- Townsend M, Shankar GM, Mehta T, Walsh DM, Selkoe DJ. Effects of secreted oligomers of amyloid beta-protein on hippocampal synaptic plasticity: a potent role for trimers. *J Physiol*. 2006; 572:477–492. [PubMed: 16469784]
- Tyan, S-H.; Midthune, B.; Eggert, S.; Dickstein, DL.; Koo, EH. Role of Amyloid Precursor proteins and Abeta-induced synaptic dysfunction; Abstract, International Conference on Alzheimer's Disease; Honolulu, HI. 2010. Abstract # 1962.
- van den Hurk WH, Bloemen M, Martens GJ. Expression of the gene encoding the beta-amyloid precursor protein APP in *Xenopus laevis*. *Brain Res Mol Brain Res*. 2001; 97:13–20. [PubMed: 11744158]
- von Koch CS, Zheng H, Chen H, Trumbauer M, Thinakaran G, van der Ploeg LH, Price DL, Sisodia SS. Generation of APLP2 KO mice and early postnatal lethality in APLP2/APP double KO mice. *Neurobiol Aging*. 1997; 18:661–669. [PubMed: 9461064]
- Wang P, Yang G, Mosier DR, Chang P, Zaidi T, Gong YD, Zhao NM, Dominguez B, Lee KF, Gan WB, Zheng H. Defective neuromuscular synapses in mice lacking amyloid precursor protein (APP) and APP-Like protein 2. *J Neurosci*. 2005; 25:1219–1225. [PubMed: 15689559]
- Wasco W, Gurubhagavatula S, Paradis MD, Romano DM, Sisodia SS, Hyman BT, Neve RL, Tanzi RE. Isolation and characterization of APLP2 encoding a homologue of the Alzheimer's associated amyloid beta protein precursor. *Nat Genet*. 1993; 5:95–100. [PubMed: 8220435]
- Wearne SL, Rodriguez A, Ehlenberger DB, Rocher AB, Henderson SC, Hof PR. New techniques for imaging, digitization and analysis of three-dimensional neural morphology on multiple scales. *Neuroscience*. 2005; 136:661–680. [PubMed: 16344143]
- Weyer SW, Klevanski M, Delekate A, Voikar V, Aydin D, Hick M, Filippov M, Drost N, Schaller KL, Saar M, Vogt MA, Gass P, Samanta A, Jaschke A, Korte M, Wolfer DP, Caldwell JH, Muller UC. APP and APLP2 are essential at PNS and CNS synapses for transmission, spatial learning and LTP. *EMBO J*. 2011; 30:2266–2280. [PubMed: 21522131]
- Wong PC, Zheng H, Chen H, Becher MW, Sirinathsinghji DJ, Trumbauer ME, Chen HY, Price DL, Van der Ploeg LH, Sisodia SS. Presenilin 1 is required for Notch1 and Dll1 expression in the paraxial mesoderm. *Nature*. 1997; 387:288–292. [PubMed: 9153393]
- Young-Pearse TL, Bai J, Chang R, Zheng JB, LoTurco JJ, Selkoe DJ. A critical function for beta-amyloid precursor protein in neuronal migration revealed by in utero RNA interference. *J Neurosci*. 2007; 27:14459–14469. [PubMed: 18160654]
- Young-Pearse TL, Chen AC, Chang R, Marquez C, Selkoe DJ. Secreted APP regulates the function of full-length APP in neurite outgrowth through interaction with integrin beta1. *Neural Dev*. 2008; 3:15. [PubMed: 18573216]
- Zheng H, Jiang M, Trumbauer ME, Sirinathsinghji DJ, Hopkins R, Smith DW, Heavens RP, Dawson GR, Boyce S, Conner MW, Stevens KA, Slunt HH, Sisodia SS, Chen HY, Van der Ploeg LH. beta-Amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity. *Cell*. 1995; 81:525–531. [PubMed: 7758106]

Figure 1. Deletion of APLP2 does not affect spine density in primary culture

A) Representative images of cultured primary hippocampal neurons that were transfected with eGFP at DIV 15–18, fixed and imaged one day post transfection. Insets show the segments in the secondary dendrites that were quantified for spine numbers after magnification. B) Quantification of spine numbers from cultured neurons shown in (A). There were no differences in spine density between the two genotypes (wt: 41 neurons from $n = 11$ mice, APLP2^{-/-}: 56 neurons from $n = 13$ mice).

Figure 2. Aged APLP2^{-/-} mice exhibit normal spine density, volume and morphometry
 A) Representative 3-dimensional reconstructions of CA1 pyramidal dendritic segments from 10–12 month old wt and APLP2^{-/-} mice. B) Automated analysis of spine density displayed no changes in total spine density or in percentage of spine type per dendritic segment (5 wt and 7 APLP2^{-/-} mice, at least 5 neurons per mouse and at least 3 dendritic segments per neuron). C) Further morphometric analysis of spine volume revealed comparable mean spine volumes amongst spine types.

Figure 3. Morphological alterations and dendritic length are not affected by the absence of APLP2 in aged animals *in vivo*

Representative examples of 3D reconstructions from pyramidal layer CA1 neurons in 10-month-old wt control (A) and APLP2^{-/-} (B) mice. Reconstructed cells were obtained using NeuroExplorer (MBF) and rotated about the principal axis to depict apical and basal dendritic orientation. Total dendritic length (C), Sholl analyses (dendritic length per radial distance from soma (D)) and intersections per radial distance from the soma were compared from apical dendrites of CA1 pyramidal cells of APLP2^{-/-} mice and their wt littermates at 10–12 months of age revealing no significant difference between genotype. Cells were traced using Neurolucida and quantified using NeuroExplorer software (n=5 wt and 7 APLP2^{-/-} mice). Values represent mean ± SEM.

Figure 4. Deletion of APLP2 does not alter basal synaptic transmission in young or aged mice
 A) Scatter plots of the fEPSP amplitude versus fiber volley for 1–2 month old and 10–12 month old APLP2^{-/-} and wt mice, fit by linear regression, demonstrated no significant difference in the input/output ratio between genotypes of young (A) or aged (B) animals (A; 14 wt, 15 APLP2^{-/-} slices from 10 and 12 animals, respectively, B; n=11 wt, n=12 APLP2^{-/-} slices from 6 and 7 animals respectively). Presynaptic release probability was examined by eliciting a second pulse 50 ms after the first pulse. No significant differences were found between genotypes at 1–2 months of age (C; 14 wt, 15 APLP2^{-/-} slices from 10 and 12 animals, respectively) or 10–12 months of age (D; 11 wt, 12 APLP2^{-/-} slices from 6

and 7 animals, respectively). Sample tracings located above the graph. Values represent mean $\pm$ SEM.

Figure 5. APLP2^{-/-} mice show normal LTP in young and aged mice

After a 20-minute baseline, LTP was induced by four 1-s tetanic stimuli delivered 5 minutes apart, each at 100 Hz. The percent increase in response after the tetanus was given was compared between the APLP2^{-/-} mice and littermate animals at 1–2 months of age (A; 14 wt, 15 APLP2^{-/-} slices from 10–12 animals/genotype) and at 10–12 months of age (B; 11 wt, 12 APLP2^{-/-} slices from 6–7 animals/genotype). No significant difference was found across genotype or age group. Sample tracings to the right of each figure. Values represent mean $\pm$ SEM.