Deficits in Synaptic Transmission and Learning in Amyloid Precursor Protein (APP) Transgenic Mice Require C-Terminal Cleavage of APP

Michael J. Saganich,^{1*} Brock E. Schroeder,^{2*} Veronica Galvan,³ Dale E. Bredesen,³ Edward H. Koo,² and Stephen F. Heinemann¹

¹Salk Institute for Biological Studies, La Jolla, California 92037, ²Department of Neurosciences, University of California at San Diego, La Jolla, California 92037, and ³Buck Institute for Age Research, Novato, California 94945

Synaptic dysfunction has been shown to be one of the earliest correlates of disease progression in animal models of Alzheimer's disease. Amyloid- β protein ($A\beta$) is thought to play an important role in disease-related synaptic dysfunction, but the mechanism by which $A\beta$ leads to synaptic dysfunction is not understood. Here we describe evidence that cleavage of APP in the C terminus may be necessary for the deficits present in APP transgenic mice. In APP transgenic mice with a mutated cleavage site at amino acid 664, normal synaptic transmission, synaptic plasticity, and learning were maintained despite the presence of elevated levels of APP, $A\beta_{42}$, and even plaque accumulation. These results indicate that cleavage of APP may play a critical role in the development of synaptic and behavioral dysfunction in APP transgenic mice.

Key words: $A\beta$ -peptide; potentiation; Schaffer collateral terminals; synaptic plasticity; hippocampus; learning memory; spatial memory; LTP; amyloid; Alzheimer's disease

Introduction

Alzheimer's disease (AD), the most common dementing disorder in the elderly, is characterized pathologically by the presence of senile plaques and neurofibrillary tangles. The pathogenesis of Alzheimer's disease is still unclear in many respects; however, evidence suggests that amyloid- β protein ($A\beta$) plays an important and potentially causal role. The "amyloid cascade hypothesis" asserts that extracellular $A\beta$ accumulation leads to a series of events culminating in the constellation of problems associated with AD. Interestingly, although $A\beta$ is itself cytotoxic, human brain analysis has shown that cell death is not well correlated with disease progression (Terry et al., 1991). Instead, increasing evidence has pointed to the importance of synaptic health in the development of the disease phenotype. Changes in the density of the presynaptic marker synaptophysin are better correlated with disease progression than amyloid plaque load or cell death (Terry et al., 1991). This idea has been confirmed by deficits in electro-

physiological measures of synaptic communication and plasticity in transgenic mouse models of amyloid deposition (Chapman et al., 1999; Hsia et al., 1999; Giacchino et al., 2000; Fitzjohn et al., 2001), changes that occur before plaque accumulation or cell death. Furthermore, lending weight to a causal and direct role, amyloid-induced deficits in long-term potentiation (LTP) can be reversed by passive immunization with $A\beta$ -specific antibodies (Klyubin et al., 2005).

Despite the abundance of evidence implicating synapses as prime targets of $A\beta$, little is known about the mechanistic pathway leading downstream from $A\beta$ to synaptic dysfunction or damage. Several groups have previously demonstrated *in vitro* the potential importance of the C terminus of amyloid precursor protein (APP), including evidence that certain cleavage products may regulate transcription, influence gene expression, and play a role in cell survival (Cao and Sudhof, 2001; Galvan et al., 2002; Kim et al., 2003). For example, it has been demonstrated previously that APP contains a consensus site for caspase cleavage at D664 (Lu et al., 2000), and *in vitro* experiments have shown that the resultant fragment (termed C31) causes cell death in neurons. Several additional findings point to the functional importance of this cleavage. First, $A\beta$ application increases cleavage of APP at D664. Second, prevention of cleavage via a D664A mutation significantly reduces $A\beta$ -induced cell death (Lu et al., 2003a). Last, cleavage of APP at D664 is increased in the brains of AD patients (Zhao et al., 2003) and in APP transgenic mice (Galvan et al., 2006). These findings suggest a model that extends the $A\beta$ hypothesis to include the downstream toxic effects of $A\beta$ being mediated, at least in part, through cleavage of APP at D664.

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*M.J.S. and B.E.S. contributed equally to this work.

Correspondence should be addressed to Dr. Michael J. Saganich, Salk Institute for Biological Sciences, 10010 North Torrey Pines Road, La Jolla, CA 92037. E-mail: saganich@salk.edu.

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Recent histological evidence has shown that prevention of cleavage of APP at D664, by the D664A mutation, may prevent synaptophysin loss in APP transgenic mice (Galvan et al., 2006). This evidence suggests that cleavage at D664 might have important functional consequences on synaptic function and synaptotoxicity in APP transgenic mice. In the present study, we describe functional evidence that C-terminal cleavage of APP at amino acid 664 (APP695 numbering) is necessary for the synaptic transmission, synaptic plasticity, and behavioral deficits present in APP transgenic mice.

Materials and Methods

Animals

The generation of the two established lines of APP transgenic mice were examined in this study; PDAPP(J9) and PDAPP(J20) has been described previously (Hsia et al., 1999). Briefly, these mice contain human APP carrying two familial AD mutations, Swedish (K670N, M671L) and Indiana (V717F) downstream from the platelet-derived growth factor β promoter. D664A mice (lines B21 and B254) were created by introducing the D664A mutation into the same human APP minigene (Galvan et al., 2006). Transgenic mice were heterozygous with respect to the transgene. Mice were maintained on a C57BL/6 background. For behavioral experiments, transgenic C57BL/6 male mice were crossed with nontransgenic female C3H/HeJ breeders, and experiments were performed on the F1 generation offspring.

Tissue analysis

Mice were killed by decapitation under halothane anesthesia. Brains were removed rapidly, snap frozen immediately, and stored at -70°C for later protein analysis or drop fixed in phosphate-buffered 4% paraformaldehyde at 4°C for 24 h for neuropathological analysis. For detection of APP and $\text{A}\beta$, frozen hippocampal tissue was homogenized in PBS (5 ml/g tissue) containing a protease inhibitor cocktail (Roche, Indianapolis, IN). Homogenate was then diluted 1:1 with 2% SDS in PBS. Homogenates were sonicated and centrifuged for 20 min at $14,000 \times g$. The supernatant was collected and total protein concentration was determined by a Micro BCA protein assay (Pierce, Rockford, IL). Western blots were probed with CT-15 (1:4000), an antibody directed at the C-terminal 15 amino acids of APP (Sisodia et al., 1993), and E7 (1:10,000), a β -tubulin recognizing antibody (Developmental Studies Hybridoma Bank, Iowa City, IA). $\text{A}\beta_{40}$ and $\text{A}\beta_{42}$ levels were determined by sandwich ELISA (IBL, Gunma, Japan). For neuropathology, 40 μm coronal hippocampal sections were mounted on glass slides and processed for thioflavin-s (Sigma, St. Louis, MO) reactivity. For D664A(B254) mice only, after physiological recordings, slices were fixed, resectioned, and mounted on glass slides for thioflavin-S staining.

In vitro slice electrophysiology

Horizontal hippocampal slices (400 μm) were made from 3- to 6-month-old PDAPP(J20), D664A(B21), and D664A(B254) and nontransgenic littermate control mice. On each day, two mice (one transgenic and one nontransgenic) were examined, with the experimenter blind to the genotype. Slices were prepared using standard methods (Contractor et al., 2003). Briefly, animals were anesthetized with isoflurane and decapitated. The brain was removed under ice-cold oxygenated sucrose-slicing artificial CSF (ACSF) containing the following: 85 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4 , 25 mM NaHCO_3 , 25 mM glucose, 75 mM sucrose, 0.5 mM CaCl_2 , and 4 mM MgCl_2 , equilibrated with 95% O_2 /5% CO_2 , which also contained 10 μM D,L-APV and 100 μM kynure-nate. Slices were incubated at 28°C for 30 min, followed by exchange of the sucrose-ACSF for an oxygenated sodium ACSF solution containing the following: 125 mM NaCl, 2.4 mM KCl, 1.2 mM NaH_2PO_4 , 25 mM NaHCO_3 , 25 mM glucose, 1 mM CaCl_2 , and 2 mM MgCl_2 , in which slices remained for at least 2 h before recording. In the recording chamber, slices were continuously perfused with oxygenated sodium ACSF containing 2 mM CaCl_2 and 1 mM MgCl_2 .

Extracellularly recorded field EPSPs (fEPSPs) were recorded in the stratum radiatum of the CA1 region of hippocampus with a glass recording pipette filled with oxygenated extracellular solution (tip resistance,

2–3 M Ω) using an Axopatch 700A amplifier (Molecular Devices, Sunnyvale, CA). fEPSPs were evoked using a concentric bipolar electrode (Frederick Haer Company, Bowdoinham, ME) positioned in the Schaffer collateral/commissural pathway. Stimuli were controlled by pClamp 9 software (Molecular Devices) and generated with an A310 Accupulser coupled to an A360 stimulation isolation unit (Warner Instruments, Hamden, CT). Basal synaptic transmission was assessed by comparing the input and output relationship of the fEPSPs recorded; input was the peak amplitude of the fiber volley, and the output was the initial slope of the fEPSP. For each animal, we measured the fiber volley amplitude and initial slope of the fEPSP responses to a range of stimulation from 150 to 800 μA , and a response curve was generated for both values. The input–output relationship was then calculated by dividing the slope of the fEPSP by the fiber volley amplitude (from each point along the linear portion on the response curve) and taking the average value. This input–output value for each stimulation level was then averaged to give single measure of basal synaptic transmission for each slice. Alternatively, we also analyzed basal synaptic transmission strength by plotting all individual fEPSP slope and fiber volley values and fit data by linear regression analysis (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). The slope of the fit data also provided a single value for synaptic transmission. LTP was induced by four tetani delivered 10 s apart, each at 100 Hz for 1 s after a 20 min baseline period. fEPSPs were monitored for 60 min after tetanus. In some cases, a theta burst was delivered in lieu of the standard tetanus. Theta burst stimulation comprised six trains given at 10 s intervals, consisting of five 40 ms bursts of 100 Hz separated by 200 ms. Paired-pulse facilitation (PPF) was elicited using an interstimulus interval of 50 ms and ratio was measured as the peak amplitude of fEPSP(2)/fEPSP(1).

For whole-cell experiments, 350 μm slices were prepared as above. Glass electrodes were filled with intracellular solution containing the following: 130 mM cesium-methanesulfonate, 4 mM NaCl, 1 mM MgCl_2 , 10 mM cesium BAPTA, 10 mM HEPES, and 10 mM QX-314 [N-(2,6-dimethylphenyl)carbonylmethyl]triethylammonium chloride] (resistance, 3–4 M Ω). Whole-cell voltage-clamp recordings were made from visually identified pyramidal cells in the CA1 region using an Axopatch 700A patch-clamp amplifier. Series resistance was continuously monitored using hyperpolarizing voltage steps generated by pClamp 9 software, and recordings were discarded if there was a $>15\%$ change during the course of the experiment. Synaptic currents were evoked using a monopolar glass electrode filled with oxygenated ACSF positioned in the stratum radiatum. Combined AMPA receptor- and NMDA receptor-mediated EPSCs were isolated using the GABA $_A$ receptor antagonists bicuculline (10 μM) and picrotoxin (50 μM) at holding potential of +40 mV. NMDA currents were isolated with the addition of 50 μM D-APV.

Morris water maze

Pretraining. All mice were first tested in a straight-swim pretraining protocol. Mice received eight trials per day for 2 d. A platform located 1 cm below the water was located opposite the start location. Latency to climb onto the platform was measured. Mice unable to complete six of eight trials in fewer than 10 s on the second day did not move on to water maze testing.

Hidden platform testing. Extramaze visual cues were hung from a curtain located around a 1.26-m-diameter circular tank. The water was made opaque by addition of nontoxic paint. A 10-cm-diameter escape platform was located 1 cm below the surface of the water, and a Polytrack (San Diego Instruments, San Diego, CA) video-tracking system was used to collect mouse movement (location, distance, and latency) data during training and probe trials. Each mouse was given eight trials per day in two blocks of four trials for 4 consecutive days. One hour after trial 32, each animal was given a 60 s probe trial. During the probe test, the platform was removed, and quadrant search times and distance in target quadrant were measured.

Visible cued testing. One day after the last hidden platform training trial, mice were trained to locate a visible-cued platform. The visible cue was a green plastic flag attached to a pole such that it was 10 cm above the platform. On each trial of the visible platform test, the platform was randomly located in one of the four quadrants. Mice were given eight

trials in blocks of four trials, and the latency to find the platform was recorded for each trial.

Statistical analysis

For all experiments, experimenters were blind with respect to genotype. Unless otherwise indicated, data were expressed as mean $\pm$ SEM. Statistical analyses were performed with the StatView 5.0 program (SAS Institute, Cary, NC). Differences among means were assessed by way of one-way ANOVA, followed by Dunnett's or Tukey–Kramer *post hoc* tests. Comparison of LTP data between different lines was tested for significance using the Kolmogorov–Smirnov test. For water maze analysis, a repeated-measures ANOVA was performed with session as the repeated measure. The null hypothesis was rejected at the 0.05 level.

Results

D664A mice maintain elevated APP expression, A β expression, and plaque formation

Two established lines of APP transgenic mice were examined in this study, PDAPP(J9) and PDAPP(J20) (Hsia et al., 1999), which contain human APP carrying two familial AD mutations. D664A mice (independent lines B21 and B254) were created (Galvan et al., 2006) by introducing the D664A mutation into the same human APP minigene used to generate the PDAPP(J20) and PDAPP(J9) lines (Fig. 1A). This mutation prohibits cleavage of APP at D664, a cleavage that is apparent in both AD patients as well as in the PDAPP(J20) line (Zhao et al., 2003; Galvan et al., 2006). We chose primarily to focus on PDAPP(J20) and D664A(B21) because of the similar magnitude of APP and A β expression at 3–6 months (Fig. 1B–D). However, to exclude the possibility that a slight difference in APP or A β expression was responsible for the results demonstrated in the D664A(B21) line below or that random insertion of the transgene may be responsible, we also replicated many of the experiments using the independent D664A(B254) line, which expresses APP at higher levels than either PDAPP(J20) or D664A(B21) and has approximately five times higher A β_{42} expression than the other transgenic lines (Fig. 1D). Consistent with the observations that neither APP processing nor A β generation are affected by the D664A mutation (Soriano et al., 2001), brain A β levels and plaque burden in brain are similar between the PDAPP(J20) and D664A(B21) lines (Fig. 1).

Deficits in basal synaptic function in PDAPP mice are absent in D664A mice

It has been shown previously that synaptic transmission deficits are one of the first altered phenotypes to appear in APP transgenic mice, substantially sooner than any evidence of amyloid deposits (Hsia et al., 1999; Giacchino et al., 2000). In considering synaptic integrity in APP transgenic mice, examination of synaptic transmission is necessary because histological determinations of synaptic numbers using markers such as synaptophysin do not address function and may in fact overestimate the number of functional synapses present in the brain (Wojtowicz et al., 1991; Isaac et al., 1995; Tong et al., 1996). In this study, extracellularly recorded fEPSPs were used to assess the strength of basal synaptic transmission at the Schaffer collateral to CA1 synapse in acute hippocampal slices from 3- to 6-month-old PDAPP(J20), D664A(B21), D664A(B254), and littermate control mouse brains. The strength of this connection was quantified for each slice by measuring the fEPSP slope and dividing this value by the corresponding fiber volley amplitude at each input stimulation level. The fiber volley amplitude is proportional to the number of axons activated, allowing for an independent measurement of input strength and offsetting artifacts introduced by different

Figure 1. Transgenic lines, expression of APP transgene, A β_{42} levels, and plaque deposition in transgenic mouse lines. **A**, Diagram of PDAPP (J9 and J20) and D664A(B21 and B254) transgenes, including familial Alzheimer's mutations (FAD) and location of D664A mutation. TMD, Transmembrane domain. **B**, Cortical brain extracts from 3- to 6-month-old mice were used for Western blot analysis of relative expression levels of APP protein using the antibody CT-15. NTg, Nontransgenic. **C**, ELISA detection of A β_{40} levels in individual 3- to 6-month-old mice are shown for all lines of mice examined (weight per 100 mg of wet weight brain tissue). **D**, ELISA detection of A β_{42} in individual 3- to 6-month-old transgenic mice (averages noted with horizontal lines; weight per 100 mg of wet weight tissue). Note the large increase in average A β_{42} levels in transgenic line D664A(B254) (** $p < 0.01$). This is particularly apparent between 3- and 6-month-old animals (6 month animals are noted by } symbol). **E**, Thioflavin-S staining of amyloid deposits in 12-month-old nontransgenic control (top) PDAPP(J20) (middle) and D664A(B21) (bottom) hippocampal sections. Magnification, 4 $\times$. Scale bar, 800 μ m.

placement of the stimulating and recording electrodes from slice to slice (Hsia et al., 1999; Fitzjohn et al., 2001).

It has been shown previously that 2- to 4-month-old PDAPP(J9) mice, which express lower levels of hAPP and A β_{42} than PDAPP(J20) mice (Fig. 1B,D), suffer from severe deficits in synaptic function (Hsia et al., 1999). As expected, 3- to 6-month-old PDAPP(J20) mice also showed deficits in basal synaptic transmission. Individual field responses from PDAPP(J20) slices were on average smaller in amplitude and easily distinguishable from control mice (Fig. 2A). Basal synaptic transmission from slices prepared from PDAPP(J20) mice was $65 \pm 7\%$ of control levels (Fig. 2A,D) ($p < 0.001$; $n = 16$ –24 slices from 7–9 animals). This deficit is clearly demonstrated when fEPSP slope is plotted against fiber volley amplitude (Fig. 2A) (supplemental Fig. 1A3, available at www.jneurosci.org as supplemental material) or when examining average fEPSP slope values alone (supplemental Fig. 1A1, available at www.jneurosci.org as supple-

Figure 2. C-terminal cleavage of PDAPP transgene is necessary for deficits in basal synaptic transmission in APP transgenic mice. Basal synaptic transmission was assayed by measuring extracellular field potentials (fEPSPs) within the stratum radiatum of the CA1 region of the hippocampus evoked by a bipolar electrode placed at the CA3–CA1 border. For each level of stimulation, the maximum amplitude of the presynaptic fiber volley (FV; arrow) and the initial slope of the fEPSP were measured (see also supplemental Fig. 1, available at www.jneurosci.org as supplemental material). **A**, Basal synaptic transmission is impaired in PDAPP(J20) animals (left). Representative responses from nontransgenic (NTg) control and PDAPP(J20) animals show a large reduction in fEPSP in PDAPP(J20) animals. Note the large input (see inset with expanded timescale) needed to elicit a relatively small EPSP in transgenic PDAPP(J20) mice. Average fEPSP slopes of nontransgenic (filled squares) and PDAPP(J20) (open circles) plotted as a function of the average fiber volley amplitude (right). **B**, D664A(B21) animals (open circles) show no impairments in basal synaptic transmission compared with littermate controls (filled squares). **C**, Even D664A(B254) animals, which express the highest levels of the PDAPP transgene, show no gross impairment in fEPSP. **D**, Average basal synaptic transmission levels of transgenic PDAPP(J20), B21, and B254 lines (open circles) normalized to nontransgenic littermate controls (filled squares) demonstrating prevention of deficits by the D664A mutation in the PDAPP transgene. $^{**}p < 0.001$.

mental material). Additional characterization of the PDAPP(J20) line showed no significant changes in PPF or AMPA/NMDA ratio compared with control mice (supplemental Fig. 2, available at www.jneurosci.org as supplemental material), the latter in con-

Figure 3. Histological detection of plaques in 6-month-old D664A(B254) mice. After physiological recordings, slices from D664A(B254) ($n = 5$) and nontransgenic (NTg) control ($n = 5$) animals were fixed, resectioned, and mounted on glass slides for thioflavin-S processing. Higher APP and $A\beta_{42}$ expression in D664A(B254) transgenic mice resulted in the earlier development of thioflavin-S-sensitive amyloid deposits in the hippocampus. Plaques were most notable in the dentate gyrus (DG, top) and the CA1 subregion (Sub, bottom) of the hippocampus. No staining was observed in nontransgenic control slices. Scale bar, 300 μ m.

trast to a previous characterization of PDAPP mice (Hsia et al., 1999).

In contrast to the PDAPP(J20 or J9) animals, no change in basal synaptic function was observed in 3- to 6-month-old D664A(B21) animals (Fig. 2B,D) ($p > 0.55$; $n = 18$ –20 slices from 7–9 animals). Large fEPSPs were easy to evoke and were indistinguishable from nontransgenic control mice. D664A(B21) slices showed no changes in the average fEPSP slope, fiber volley, or when fEPSP slopes were normalized to fiber volley (Fig. 2B) (supplemental Fig. 1B, available at www.jneurosci.org as supplemental material). No change in PPF was observed in D664A(B21) animals compared with control (data not shown).

To account for the possibility of line-specific protective effects attributable to incorporation of the transgene in the D664A(B21) line, we also examined synaptic function in the higher expressing independent D664A(B254) transgenic line. Resembling D664A(B21) mice, the magnitudes of individual field responses from slices prepared from 3- to 6-month-old D664A(B254) animals showed no obvious deficits (Fig. 2C), and, although the average input–output value was reduced by $\sim 12\%$, this was not a statistically significant change in basal synaptic function (Fig. 2D) ($p > 0.09$; $n = 27$ –30 slices from 9–10 animals). No significant changes were seen in the average fEPSP slope or average fiber volley amplitude (supplemental Fig. 1C, available at www.jneurosci.org as supplemental material) ($p > 0.05$; $n = 27$ –30 slices). These results are particularly noteworthy considering the very high levels of APP and $A\beta_{42}$ in D664A(B254) animals (Fig. 1B,D). In fact, when the basal synaptic transmission results were analyzed using only data from 6-month-old D664A(B254) mice [when $A\beta_{42}$ levels were markedly higher than the other lines (Fig. 1D)], there was still no significant change in synaptic transmission. Furthermore, when these same hippocampal slices from 5- to 6-month-old D664A(B254) animals were processed for thioflavin-S after our recordings, amyloid deposits were already detectable (Fig. 3). In comparison, plaques are not detectable at 6 months of age in PDAPP(J9), PDAPP(J20), or D664A(B21) mice

(data not shown). This remarkable finding, namely, the absence of significant deficits in synaptic transmission even in the presence of extremely high $A\beta_{42}$ levels and early plaque formation, is consistent with the conclusion that the early loss in basal synaptic transmission normally observed in PDAPP mice requires a functional caspase cleavage site in the C terminus of APP.

Deficits in synaptic plasticity (LTP) in PDAPP mice are absent in D664A mice

Synaptic plasticity and LTP are thought to underlie the experience-dependent modification of behavior (Malenka and Bear, 2004). Deficits in synaptic plasticity in the hippocampus of AD model mice have been reported previously by multiple groups (Chapman et al., 1999; Larson et al., 1999; Giacchino et al., 2000; Oddo et al., 2003), although not in all studies (Hsia et al., 1999). In the present study, PDAPP(J20) mice had significant deficits in LTP at the Schaffer collateral–CA1 synapse compared with control mice ($p < 0.0001$). One hour after LTP induction, a $165 \pm 5\%$ ($n = 10$) increase in the average fEPSP slope was observed in nontransgenic mice compared with a $135 \pm 9\%$ ($n = 6$) increase in PDAPP(J20) animals (Fig. 4A). In contrast, we saw no reduction in LTP in either D664A(B21) mice ($n = 6$ per group) or the higher APP expressing D664A(B254) mice ($n = 7$ per group) when compared with control mice (Fig. 4B–D). Similar results also were observed after theta burst tetanus stimulation protocol (data not shown). One potential concern in these data are whether the lack of a potentiated response in PDAPP(J20) transgenic animals can be explained simply by a reduction in “synaptic drive.” Two points argue against this interpretation: first, the baseline drive for slices from transgenic and nontransgenic mice were set at identical levels before tetanus stimulation; second, examination of the fEPSP response immediately after tetanus stimulation revealed that PDAPP(J20) mice were able to be potentiated to similar levels as control. It was specifically in the ability to maintain this potentiated state that the PDAPP(J20) animals showed a deficiency (Fig. 4A).

Deficits in learning (Morris water maze) in PDAPP mice are absent in D664A mice

Our observation that cleavage of APP at residue D664 appears to be necessary for the early loss of synaptic connections and changes in synaptic plasticity observed in PDAPP mice prompted us to explore the functional consequences of this mutation at the behavioral level. Numerous APP transgenic mouse lines have been shown to have behavioral learning deficits, and, importantly for this study, both PDAPP(J9) and PDAPP(J20) lines have been shown previously to have learning deficits by 6–7 months of age (Raber et al., 2000; Palop et al., 2003). Using the hidden platform Morris water maze learning paradigm, we chose to examine animals in two age groups: 3–4 months, before plaque formation but

Figure 4. D664A mutation prevents LTP deficits observed in PDAPP(J20) animals at the Schaffer collateral to CA1 synapse. After a 20 min baseline, LTP was induced by four tetani delivered 10 s apart, each at 100 Hz for 1 s. fEPSPs were monitored for 60 min after tetanus. Representative traces are superimposed from the last 10 min of the baseline period and 55–60 min after LTP induction. **A–C**, Average time course of LTP in PDAPP(J20), D664A(B21), and D664A(B254) transgenic mice (open circles) compared with nontransgenic control animals (filled squares). LTP in PDAPP(J20) animals was significantly lower compared with control (**A**). In contrast to PDAPP(J20) animals, LTP is normal in both D664A(B21) (**B**) and the higher expressing D664A(B254) lines (**C**). **D**, Cumulative probability distribution of LTP measured as the percentage of potentiation 55–60 min after tetanus compared with baseline period. LTP in PDAPP(J20) animals was significantly lower than D664A(B21), D664A(B254), or nontransgenic (NTg) controls. Calibration: 0.25 mV, 10 ms.

while synaptic transmission deficits are readily apparent in PDAPP(J20) mice, and 8–12 months, when both PDAPP(J20) and D664A(B21) show plaque deposition (Fig. 1E). At the 3- to 4-month-old time point, PDAPP(J20) mice showed longer distance-to-platform measurements in the last four sessions, but the difference did not reach statistical significance (Fig. 5A) ($p = 0.11$; $n = 9$ –10 per group). D664A(B21) animals learned normally, with both latency to find the platform (data not shown) and distance to the platform showing similar values as nontransgenic littermates (Fig. 5B) ($p = 0.21$; $n = 10$ per group). By 8–12 months of age, PDAPP(J20) mice are significantly impaired in learning to find the hidden platform (Fig. 5C) ($p < 0.0001$; $n = 9$ –10 per group), findings consistent with a previous study (Palop et al., 2003). Memory retention, as measured by probe trial 1 h after the last acquisition session, was not significantly different in any group (data not shown). In a separate visible platform test, PDAPP(J20) mice showed no deficits (supplemental Fig. 3A,B, available at www.jneurosci.org as supplemental material), indicating that the ability to use visual cues as guides was not deficient in these mice. Similar to our findings examining synaptic transmission, D664A(B21) mice did not show any impairment in learning at 8–12 months of age (Fig. 5D) ($p > 0.40$; $n = 11$ –12 per group). Thus, despite the presence of elevated levels of $A\beta_{42}$ and amyloid deposits comparable with PDAPP(J20) animals (Fig. 1D,E), D664A(B21) mice do not show the same behavioral deficits that have previously been or are here described in PDAPP mice with either lower (J9) or higher (J20) $A\beta_{42}$ levels.

Figure 5. D664A mutation prevents learning deficits observed in PDAPP(J20) animals. Animals were trained for 4 d (2 sessions per day) in a hidden platform Morris water maze. Distance to platform (in centimeters) and latency to platform were measured; however, only distance is shown. **A**, At 3–4 months, PDAPP(J20) mice appeared to show longer distance-to-platform measurements in the last four sessions, but the data did not reach statistical significance. **B**, D664A(B21) animals learn normally. **C**, At 8–12 months, PDAPP(J20) mice show significant learning impairments (** $p < 0.0001$; $n = 19$). Probe trials were not significantly different between groups at either age (data not shown). **D**, At 8–12 months, D664A(B21) mice do not show any learning impairment. NTg, Nontransgenic.

Discussion

The data presented herein describe evidence implicating cleavage of APP at D664 as an important participant in the mechanism of synaptic dysfunction and behavioral disturbance in a model of amyloid-associated pathology in mice. We have shown that, despite the presence of high amounts of $A\beta_{40}$, $A\beta_{42}$, and corresponding amyloid deposits, a single amino acid replacement, that prohibits cleavage at D664, leads to a protection from the predicted dysfunction in synaptic transmission and learning behavior. Specifically, D664A(B21) mice express APP and $A\beta_{42}$ at levels between the PDAPP(J9) and PDAPP(J20) lines and yet are protected from the synaptic dysfunction and learning deficits present in both of those lines. Furthermore, the even higher expressing line, D664A(B254), also shows preservation of synaptic function, even at an age when plaque accumulation has begun. Whether the deficits in the PDAPP(J20) mice are attributable to APP or $A\beta$ overexpression cannot be ascertained from the experiments in this study; however, several groups have demonstrated that $A\beta$ immunotherapy reverses cognitive deficits in APP transgenic mice (Dodart et al., 2002; Kotilinek et al., 2002; Wilcock et al., 2004), suggesting that $A\beta$ likely plays an important role in the deficits observed in APP-overexpressing transgenic mice.

The ability of $A\beta$ to induce dysfunction is well established at the cellular, synaptic, and behavioral level (Walsh and Selkoe, 2004). Although much research has focused on the β -secretase and γ -secretase cleavage of the APP protein and the creation and role of $A\beta$, several groups have suggested that the C terminus of APP may also play a role in AD (Cao and Sudhof, 2001; Galvan et al., 2002; Kim et al., 2003). Critical to the present study is recent evidence showing that the D664A mutation indeed prevents cleavage at D664 and thus the formation of the C31 fragment *in vivo* (Galvan et al., 2006), confirming previous *in vitro* findings

(Lu et al., 2003b). This study was also in agreement with our data (Fig. 1) that the D664A mutation does not affect formation or accumulation of $A\beta$. Finally, the study provided preliminary evidence suggesting that cleavage at D664 may be playing a role in synaptic health, because the loss in synaptophysin staining in PDAPP(J20) transgenic mice was prevented in D664A(B21) mice.

Reduction in synaptophysin puncta, commonly used for measuring synaptic loss, has been reported in human AD patients (Terry et al., 1991; Masliah et al., 2001), as well as in multiple APP transgenic lines (Hsia et al., 1999; Mucke et al., 2000; Spire et al., 2005), although not in all studies (for example, see King and Arendash, 2002). However, in addition to inherent difficulties in using immunofluorescence as a quantitative technique, this method also requires the assumption that every puncta is a mature synapse with a corresponding postsynaptic specialization and functionality. A more rigorous histological study combining both synaptophysin staining and electron microscopy of synapses recently confirmed synaptic loss in the hippocampus of APP transgenic mice compared with age-matched controls (Buttini et al., 2005).

Because of the difficulty in interpreting the functional significance of histological synaptic measurements, however, synaptic function has also been directly measured electrophysiologically in at least five independent APP transgenic mouse lines, including PDAPP (Indiana) (Hsia et al., 1999; Larson et al., 1999; Giacchino et al., 2000; Hartman et al., 2005), PDAPP(J9) (Hsia et al., 1999), Tg2576 (Chapman et al., 1999; Fitzjohn et al., 2001), APP London (Moechars et al., 1999; Dewachter et al., 2002), and the 3xTg-AD line (Oddo et al., 2003). Although the interpretation of these results can be complicated by the use of different physiological techniques, expression levels of transgenes and ages and background of animals, a clear consensus has emerged that synaptic function is disrupted in APP-overexpressing transgenic mice. Moreover, this dysfunction often occurs before histological detection of plaques. Two assays for measuring synaptic function are most commonly used: first, measurement of the magnitude of the fEPSPs after evoked stimulation, which is often referred to as “basal synaptic transmission,” and, second, measurement in activity-dependent changes in synaptic strength or LTP.

Reductions in basal synaptic transmission in PDAPP (Indiana) mice have been shown clearly *in vitro* (Hsia et al., 1999; Larson et al., 1999) as well as *in vivo* (Giacchino et al., 2000). Deficits also have been observed in APP London mice (Moechars et al., 1999), as well as Tg2576 (Swedish) APP transgenic mice (Fitzjohn et al., 2001). An analysis of these data show that the level of dysfunction in basal synaptic transmission often increases with the animals' age and concurrently with $A\beta$ levels and amyloid deposits. It is therefore not surprising to see studies reporting smaller changes or no changes in basal synaptic transmission when experimenting on younger animals (Larson et al., 1999; Giacchino et al., 2000). Although there is consensus that basal synaptic transmission is reduced in APP transgenic mice, one study showed no change (Chapman et al., 1999). One possible explanation for this discrepancy is the method used in the measurement and analysis of basal synaptic transmission. As explained above, we feel normalization of fEPSP using the fiber volley amplitude, correcting for alterations in electrode/stimulator location, is critical for accurate interpretation of basal synaptic transmission data.

Deficits in the ability to induce long-term synaptic changes have also been observed in several different APP transgenic lines. LTP deficits in PDAPP (Indiana) mice (Larson et al., 1999; Giac-

chino et al., 2000; Hartman et al., 2005), APP London (Moechars et al., 1999; Dewachter et al., 2002), Tg2576 (Chapman et al., 1999), and the 3xTg-AD line (Oddo et al., 2003) all have been reported. Exceptions include two studies in which no deficits were uncovered; these include studies of PDAPP (Indiana) mice (Hsia et al., 1999) and Tg2576 mice (Fitzjohn et al., 2001), both lines in which LTP deficits have been noted by other groups. Discrepancies in the field regarding LTP in APP transgenic mice may be attributable to differences in LTP induction protocols or the age of the mice when the experiments were conducted (as with basal synaptic transmission, LTP deficits often increase with age). Additionally, because basal synaptic transmission is affected in APP transgenic mice (in most of these cases), it is unclear how reduced synaptic drive might influence LTP or whether the experimenter can compensate for this fact via normalization of baseline drive.

PDAPP(J9) mice were reported previously to have deficits in basal synaptic transmission, even at younger (2–4 months) ages (Hsia et al., 1999). However, LTP was not investigated in the J9 line [only PDAPP (Indiana) mice were studied]. PDAPP(J20) mice, which were examined in this manuscript, were manufactured by the introduction of the same transgene as the J9 line; however, APP and A β expression levels are much higher (presumably attributable to increased copy number). Therefore, it was not surprising that these animals showed deficits in basal synaptic transmission (Fig. 2). Moreover, we also demonstrated a significant reduction in LTP in these mice, a novel finding (Fig. 4).

Many APP transgenic mouse lines have been shown to have behavioral learning deficits, including both the PDAPP(J9) and the PDAPP(J20) lines (Raber et al., 2000; Palop et al., 2003). In general agreement with a recent study, despite a different protocol and analysis (Galvan et al., 2006), we found that PDAPP(J20) mice showed learning deficits, whereas D664A(B21) mice learned a Morris Water Maze task normally, despite their elevated APP and A β levels. Memory retention, in the form of a probe trial, did not reach significance between groups. In examining the data, it appeared that the PDAPP(J20) mice performed at “normal” levels, perhaps reflecting the short interval between the last training session and the probe trial session. Nevertheless, our data reveal an important deficit in learning, which is prevented in the D664A(B21) line.

Considering the strong evidence for disruption of synaptic function in APP- and A β -overexpressing mice, it is important to note that a single amino acid substitution, D664A, which prohibits cleavage at D664, was able to normalize phenotypes that have been shown to be and would be predicted to be abnormal (Figs. 2, 4, 5). In addition, to avoid issues of transgene expression level, we chose to characterize two separate lines of D664A mice: the D664A(B21), which had similar levels of APP expression as the PDAPP(J20) mice with which they were compared, and a second D664A(B254) line, which expressed much higher levels of the APP transgene, presumably making prevention of any AD phenotypes much more difficult.

On the surface, the present data might appear to challenge the role of A β in the synaptic toxicity present in AD mouse models.

Figure 6. Schematic of proposed role of A β and D664 cleavage in the development of cellular dysfunction. A β may function, in part, by binding to APP and causing APP to dimerize, an event that leads to the recruitment of caspases and cleavage of APP at D664. This model demonstrates a possible mechanistic pathway leading from A β to caspase cleavage at D664 to synaptic dysfunction.

On the contrary, our data strengthen a previously proposed hypothesis implicating both A β and APP in a mechanistic pathway underlying cellular dysfunction (Lu et al., 2003). First, it has been shown that loss of APP attenuates A β toxicity (Lorenzo et al., 2000). Second, we have previously proposed a model (Fig. 6) based on cell culture experiments that A β can initiate a series of events by binding to and causing the APP transmembrane protein to form a complex, an event that may be necessary for one component of A β -induced cell death (Lorenzo et al., 2000; Lu et al., 2003; Shaked et al., 2006). In these studies, the dimerization of APP has been shown to lead to the recruitment of caspase 8 and the cleavage of APP at D664 (Lu et al., 2003). Furthermore, the dimerization of APP appears to be a critical step, given that artificial crosslinking of APP results in cytotoxicity even in the absence of soluble A β . Dimerization alone, however, is not sufficient for cytotoxicity because the D664A mutation (abrogating cleavage at D664 and C31 formation) attenuates cell death caused by either A β or artificial dimerization of APP. The inference from all of these studies is that the D664A mutation leads to a loss of caspase cleavage at that position, reducing generation of C31 and, in so doing, lowering cell death. However, the precise mechanism by which the D664A mutation attenuates cytotoxicity in our studies remains to be defined.

While in a cell culture environment, A β treatment leads to D664 cleavage and cell death, our data suggest that, *in vivo*, this cleavage event leads to an initial loss or toxicity to functional synaptic connections. This dysfunction, as opposed to losses in overall cell numbers, is consistent with previous observations in AD patients and APP transgenic mice that synaptotoxicity occurs well before other neuropathological events (Hsia et al., 1999; Giacchino et al., 2000). Our data suggest that preventing cleavage of the PDAPP transgene at position D664 renders the APP protein and its cleavage products ineffective at disrupting synaptic transmission. We propose, therefore, that A β may serve as an upstream factor, causing the dimerization of APP, which leads to cleavage of APP at the D664 site. The fact that D664 cleavage is known to occur in AD patients as well as in APP transgenic mice (Zhao et al., 2003; Galvan et al., 2006) lends additional support to the hypothesis that this cleavage may be an important downstream event in A β -induced synaptic dysfunction.

With cleavage of APP at D664 now well established *in vitro* and *in vivo*, one logical hurdle remains to be elucidated: if prevention of cleavage at D664 is protective, one has to assume that cleavage at D664 (and creation of the C31 fragment) has a functional role in synaptic and/or cellular dysfunction. A number of groups have speculated on potential mechanistic roles for C31 and other APP C-terminal cleavage products (Cao and Sudhof, 2001; Galvan et al., 2002; Kim et al., 2003; Shaked et al., 2006), with much focus on the potential for a role as a transcription factor. Another possibility is that the new domain in the remaining N-terminal fragment of APP after D664 cleavage may have a toxic function, although this is unlikely because APP deleted of the C-terminal 31 amino acids has no effect in cell culture (Lu et al., 2003).

Although our current data fit into this model pathway, the present experiments focused on the long-term effects of APP and A β overexpression. Other pathways also are likely involved in A β -induced toxicity. For example, acute effects of A β , which were not examined in the present study, have been shown to be dependent on excitatory postsynaptic receptor activity (Kamenetz et al., 2003). Even with this in mind, if prevention of D664 cleavage is indeed protective for synaptic function in the presence of A β , then uncovering the mechanism by which this cleavage contributes to dysfunction has implications both for understanding synaptic dysfunction as well as developing drugs to treat Alzheimer's disease.

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