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Fyn Kinase Modulates Synaptotoxicity, But Not Aberrant Sprouting, in Human Amyloid Precursor Protein Transgenic Mice

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Alzheimer's disease (AD), the most common neurodegenerative disorder, results in progressive degeneration of synapses and aberrant sprouting of axon terminals. The mechanisms underlying these seemingly opposing cellular phenomena are unclear. We hypothesized that Fyn kinase may play a role in one or both of these processes because it is increased in AD brains and because it is involved in synaptic plasticity and axonal outgrowth. We investigated the effects of Fyn on AD-related synaptotoxicity and aberrant axonal sprouting by ablating or overexpressing Fyn in human amyloid precursor protein (hAPP) transgenic mice.

On the *fyn*$^{+/+}$ background, hAPP/amyloid β peptide (Aβ) decreased hippocampal levels of synaptophysin-immunoreactive presynaptic terminals (SIPTs), consistent with previous findings. On the *fyn*$^{-/-}$ background, hAPP/Aβ did not affect SIPTs. SIPT reductions correlated with hippocampal Aβ levels in hAPP/*fyn*$^{+/+}$, but not hAPP/*fyn*$^{-/-}$, mice suggesting that Fyn provides a critical link between hAPP/Aβ and SIPTs. Furthermore, overexpression of Fyn exacerbated SIPT reductions in hAPP mice. We also found that the susceptibility of mice to hAPP/Aβ-induced premature mortality was decreased by Fyn ablation and increased by Fyn overexpression. In contrast, axonal sprouting in the hippocampus of hAPP mice was unaffected. We conclude that Fyn-dependent pathways are critical in AD-related synaptotoxicity and that the pathogenesis of hAPP/Aβ-induced neuronal alterations may be mechanistically heterogenous.

Key words: **Alzheimer's disease; amyloid β; Fyn kinase; synaptic deficits; signaling; sprouting; GAP-43; neurodegeneration**

Introduction

The amyloid precursor protein (APP) is expressed in many cell types and is particularly abundant in synapses. One of its metabolites, amyloid β peptide (Aβ), suppresses synaptic transmission and may participate in the regulation of neuronal activity (Hsia et al., 1999; Kamenetz et al., 2003). Mutations in the human APP (hAPP) gene that lead to increased levels of Aβ cause autosomal dominant familial Alzheimer's disease (FAD) (Selkoe and Schenk, 2003). Of several species of Aβ produced, the 42-residue form (Aβ1-42) is particularly susceptible to aggregation and is a primary constituent of neuritic amyloid plaques, pathological hallmarks of the disease (Selkoe and Schenk, 2003). Both FAD and sporadic AD are associated with a progressive degeneration of neurons and synapses (DeKosky and Scheff, 1990; Terry et al., 1999) and an aberrant sprouting of axon terminals (Geddes et al., 1985; Masliah et al., 1991; Arendt, 2001). The precise relationship between hAPP, Aβ, plaques, synaptic loss, axonal sprouting, and cognitive decline in AD is unknown.

Although amyloid plaques are a diagnostic feature of AD, growing evidence suggests that plaques may not be the primary cause of AD-related synaptic alterations and cognitive decline. In transgenic (TG) mice overexpressing hAPP/Aβ in neurons, synaptic and behavioral deficits are detectable well before plaque formation (Holcomb et al., 1999; Hsia et al., 1999; Mucke et al., 2000; Raber et al., 2000; Buttini et al., 2002; Westerman et al., 2002; Palop et al., 2003). Aβ1-42 can form small neurotoxic assemblies that act intracellularly or extracellularly and could impair neuronal and synaptic functions independent of plaques (Lambert et al., 1998; Caughey and Lansbury, 2003). Aβ interacts with a number of cell surface receptors on neurons, including integrins (Sabo et al., 1995; Bi et al., 2002), α7 nicotinic ACh receptors (Dineley et al., 2001), and the p75 neurotrophin receptor (Yaar et al., 1997). Additionally, several intracellular molecules have been implicated in Aβ-induced alterations of signaling cascades that could result in the dysfunction or degeneration of neurons (Klein, 2000; Small et al., 2001; Williamson et al., 2002; Grace and Busciglio, 2003).

One of these intracellular molecules is Fyn kinase, a member of the src family of nonreceptor tyrosine kinases. Fyn immunoreactivity (IR) is increased in AD brains (Shirazi and Wood, 1993), and genetic ablation of Fyn rendered hippocampal slices resistant to the neurotoxic effects of Aβ oligomers (Lambert et al.,

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1998). Here, we used genetic modulations of Fyn (overexpression vs ablation) to test the hypotheses that Fyn is involved in degenerative (synaptic deficits) and trophic (aberrant sprouting) effects of hAPP/Aβ.

Materials and Methods

TG mice. $hAPP_{FAD}$ TG lines J20 and J9 produce hAPP carrying Swedish (K670N, M671L) and Indiana (V717F) FAD mutations (hAPP770 numbering) (Rockenstein et al., 1995; Mucke et al., 2000). Lines were crossed for 6–10 generations onto a C57BL/6 background using mice from The Jackson Laboratory (Bar Harbor, ME). Heterozygous FYN TG mice (line N8) overexpress wild-type mouse Fyn directed by the calcium/calmodulin-dependent protein kinase IIα promoter on a C57BL/6 background (Kojima et al., 1997, 1998). $Fyn^{-/-}$ mice (Grant et al., 1992) on a 129/SVJ background were obtained from The Jackson Laboratory. Measurements were performed on gender-balanced groups. Mice were anesthetized and flush-perfused transcardially with PBS. Hemibrains were fixed in 4% phosphate-buffered paraformaldehyde or stored at −70°C.

Growth-associated protein 43 analysis. Vibratome (50 μm) or sliding microtome (30 μm) sections were avidin–biotin/immunoperoxidase stained using anti-growth-associated protein 43 (GAP-43) (1:400; Sigma, St. Louis, MO), biotinylated donkey anti-mouse (1:500; Jackson ImmunoResearch, West Grove, PA), and DAB. For quantitation of GAP-43 IR, two sections per mouse were used. In each section, the integrated optical density of staining in two areas of the outer molecular layer (OML) and two areas of the middle molecular layer (MML) of the dentate gyrus was determined by BioQuant Image Analysis (R&M Biometrics, Nashville, TN). Measurements for each area were averaged and used to calculate group means.

Synaptophysin-immunoreactive presynaptic terminal analysis. Vibratome sections (50 μm) were labeled with anti-synaptophysin (1 μg/ml; Boehringer Mannheim, Indianapolis, IN) and FITC-conjugated horse anti-mouse IgG (1:75; Vector Laboratories, Burlingame, CA) and imaged by confocal microscopy (MRC1024; Bio-Rad, Hercules, CA). For each experiment, we first determined the linear range of the fluorescence intensity of synaptophysin-immunoreactive presynaptic terminals (SIPTs) in control sections from nontransgenic (NTG) wild-type mice. This setting was then used to collect all images analyzed in the same experiment. Sections were blind coded and processed in parallel. Twelve confocal images of the molecular layer of the dentate gyrus were obtained from three sections per mouse and analyzed with NIH Image software. The area occupied by SIPTs of defined signal intensity was quantified and expressed as a percentage of the total image area, as described (Masliah et al., 1992; Buttini et al., 1999). Previous studies indicated that SIPT measurements are not significantly influenced by genetic backgrounds (Mucke et al., 1994).

Aβ ELISAs. Snap-frozen hippocampi were homogenized in guanidine buffer, and Aβ peptides were quantitated by ELISA, as described (Johnson-Wood et al., 1997).

Plaque quantitation. Vibratome sections were immunoperoxidase labeled with an Elite kit (Vector Laboratories), using biotinylated anti-Aβ1-5 (5 μg/ml, 3D6; Elan Pharmaceuticals, South San Francisco, CA) (Johnson-Wood et al., 1997) and DAB, and counterstained with 1% hematoxylin. The percentage of area of the hippocampus covered by 3D6 IR was determined with a Quantimet 570C (Leica, Deerfield, IL). Three sections were analyzed per mouse, and the average was used to calculate group means.

Statistical analyses. Statistical analyses were performed with Statview 5.0 (SAS Institute, Cary, NC). Differences between means were assessed by Student's *t* test or two-factor ANOVA, followed by the Tukey–Kramer *post hoc* test. Correlations were assessed by simple regression analysis.

Results

Modulating Fyn levels alters $hAPP_{FAD}$/Aβ-induced synaptotoxicity

We examined SIPT levels in $hAPP_{FAD}$ mice in the context of wild-type, increased, or absent expression of Fyn. First, we crossed heterozygous FYN TG mice in which wild-type mouse Fyn is overexpressed in the forebrain (Kojima et al., 1997, 1998) with heterozygous TG mice from the low $hAPP_{FAD}$/Aβ expresser line J9 (Hsia et al., 1999; Mucke et al., 2000), resulting in $fyn^{+/+}$ littermates of four genotypes: $hAPP_{low}$/FYN, $hAPP_{low}$, FYN, and NTG.

Overexpression of Fyn worsened SIPT reductions in $hAPP_{low}$ mice (Fig. 1*A*). Both $hAPP_{low}$ ($p < 0.0001$) and FYN ($p < 0.001$) had a clear effect on SIPT levels. *Post hoc* analysis revealed that SIPT levels were significantly lower in doubly TG $hAPP_{low}$/FYN mice than in singly TG $hAPP_{low}$ ($p < 0.05$) or FYN ($p < 0.001$) mice (Fig. 1*B*). Although the $hAPP_{low}$-induced SIPT reductions were relatively modest, we have previously shown that such SIPT reductions are accompanied by major reductions in synaptic transmission strength, which underscores their pathophysiological significance (Hsia et al., 1999).

Next, we crossed Fyn-deficient ($fyn^{-/-}$) mice (Grant et al., 1992) for two generations with heterozygous TG mice from the high $hAPP_{FAD}$/Aβ expresser line J20 (Mucke et al., 2000; Palop et al., 2003), yielding $hAPP_{high}$/$fyn^{+/+}$, $hAPP_{high}$/$fyn^{-/-}$, $fyn^{+/+}$, and $fyn^{-/-}$ mice. Although ablation of Fyn per se decreased SIPT levels compared with $fyn^{+/+}$ littermates (Fig. 1*C*), there was a significant interaction between the $hAPP_{high}$ and *fyn* genotype ($p < 0.0001$), indicating that $hAPP_{high}$ differentially affects presynaptic terminals in the presence or absence of wild-type Fyn. Indeed, overexpression of $hAPP_{high}$ significantly reduced SIPT levels in $fyn^{+/+}$, but not $fyn^{-/-}$, mice (Fig. 1*C*).

Modulating Fyn levels does not alter Aβ levels but changes the relationship between Aβ and SIPT

To determine whether the genetic modulation of Fyn affects the production or metabolism of Aβ, we examined hippocampal Aβ levels by ELISA. The levels of Aβ1-42 and Aβ1-x (approximates total Aβ), and Aβ1-42/Aβ1-x ratios, were comparable in 6- to 8-month-old doubly TG $hAPP_{low}$/FYN mice and singly TG $hAPP_{low}$ mice (Fig. 1*D*), suggesting that increased levels of Fyn do not alter the production or degradation of Aβ.

Aβ levels in 4- to 5-month-old $hAPP_{high}$ mice were also comparable on the $fyn^{+/+}$ and $fyn^{-/-}$ backgrounds (Fig. 1*E*). Interestingly, SIPT levels at this age correlated inversely with Aβ1-42 levels and Aβ1-42/Aβ1-x ratios in $hAPP_{high}$/$fyn^{+/+}$ mice but not in $hAPP_{high}$/$fyn^{-/-}$ mice (Fig. 1*E*), suggesting that Fyn is critical in linking Aβ with SIPT reductions.

At 4–5 months, $hAPP_{high}$ mice had no or only few plaques on the $fyn^{+/+}$ or $fyn^{-/-}$ backgrounds (data not shown). We therefore compared plaque loads in $hAPP_{high}$ mice at 8–10 months of age, when all of them had plaques. Their hippocampal plaque loads were comparable on the $fyn^{+/+}$ and $fyn^{-/-}$ backgrounds (Fig. 1*G*), suggesting that the differential effects of $hAPP_{FAD}$/Aβ on these backgrounds were not attributable to modulation of Aβ deposition.

Premature death in $hAPP_{FAD}$ mice is modulated by Fyn

Some lines of hAPP mice exhibit premature mortality (Hsiao et al., 1995; Carlson et al., 1997), but the mechanisms remain unknown. Approximately 10% of our $hAPP_{high}$/$fyn^{+/+}$ mice died before 6 months of age (Fig. 1*H*), compared with 0% of NTG $fyn^{+/+}$ controls. Although $fyn^{-/-}$ mice were slightly more susceptible to premature death than $fyn^{+/+}$ mice, ablation of Fyn improved survival in $hAPP_{high}$ mice, and $hAPP_{high}$ did not increase premature mortality on the $fyn^{-/-}$ background (Fig. 1*H*). Notably, NTG and $hAPP_{low}$ or FYN singly TG mice did not die prematurely, whereas 20% of $hAPP_{low}$/FYN doubly TG mice died during the first 6 months after birth (Fig. 1*I*).

Aberrant axonal sprouting in hAPP$_{FAD}$ mice depends on levels of hAPP$_{FAD}$/Aβ expression

In AD, the loss of presynaptic terminals is associated with aberrant axonal sprouting (Geddes et al., 1985; Masliah et al., 1991; Arendt, 2001). To determine whether these processes are linked mechanistically, we analyzed axonal sprouting in our TG models with antibodies against GAP-43, which is expressed by many types of growing axons (Benowitz and Perrone-Bizzozero, 1991). hAPP$_{high}$ mice showed prominent age-dependent axonal sprouting in the molecular layer of the dentate gyrus (Fig. 2*A*,*B*). This layer contains the dendritic arbors of the dentate granule cells and presynaptic terminals of afferent projections from other regions such as the entorhinal cortex (glutamatergic) and the basal forebrain and medial septum (cholinergic). At 4 months of age, hAPP$_{high}$ mice showed aberrant sprouting primarily in the OML (Fig. 2*B*). By 6 months, significant sprouting was observed in both the OML and the MML (Fig. 2*A*,*B*,*D*). In contrast, hAPP$_{low}$ mice had no significant increase in axonal sprouting at 6 (Fig. 2*C*,*E*) or 20 (data not shown) months, suggesting that a threshold level of hAPP$_{FAD}$/Aβ expression is required to elicit aberrant sprouting.

Aberrant axonal sprouting in hAPP$_{FAD}$ mice is independent of Fyn kinase

Because genetic ablation of Fyn prevented additional SIPT reductions (Fig. 1*C*) and eliminated the inverse correlation between SIPT and Aβ levels in hAPP$_{high}$ mice (Fig. 1*F*), we examined whether ablation of Fyn also interfered with aberrant sprouting. Ablation of Fyn did not alter either the magnitude of sprouting (Fig. 3*A*) or the age at which sprouting became evident (data not shown). In 4- to 5-month-old mice, GAP-43 in the OML was unaffected by the *fyn* genotype but was increased by hAPP$_{high}$ on both *fyn*$^{+/+}$ and *fyn*$^{-/-}$ backgrounds ($p < 0.0001$), and there was no GAP-43-related interaction between hAPP$_{high}$ and *fyn*. The magnitude of axonal sprouting in hAPP$_{high}$ mice did not correlate with hippocampal Aβ levels on *fyn*$^{+/+}$ and *fyn*$^{-/-}$ backgrounds (Fig. 3*B*). Finally, overexpression of Fyn failed to elicit aberrant sprouting in hAPP$_{low}$/FYN doubly TG mice (Fig. 3*C*).

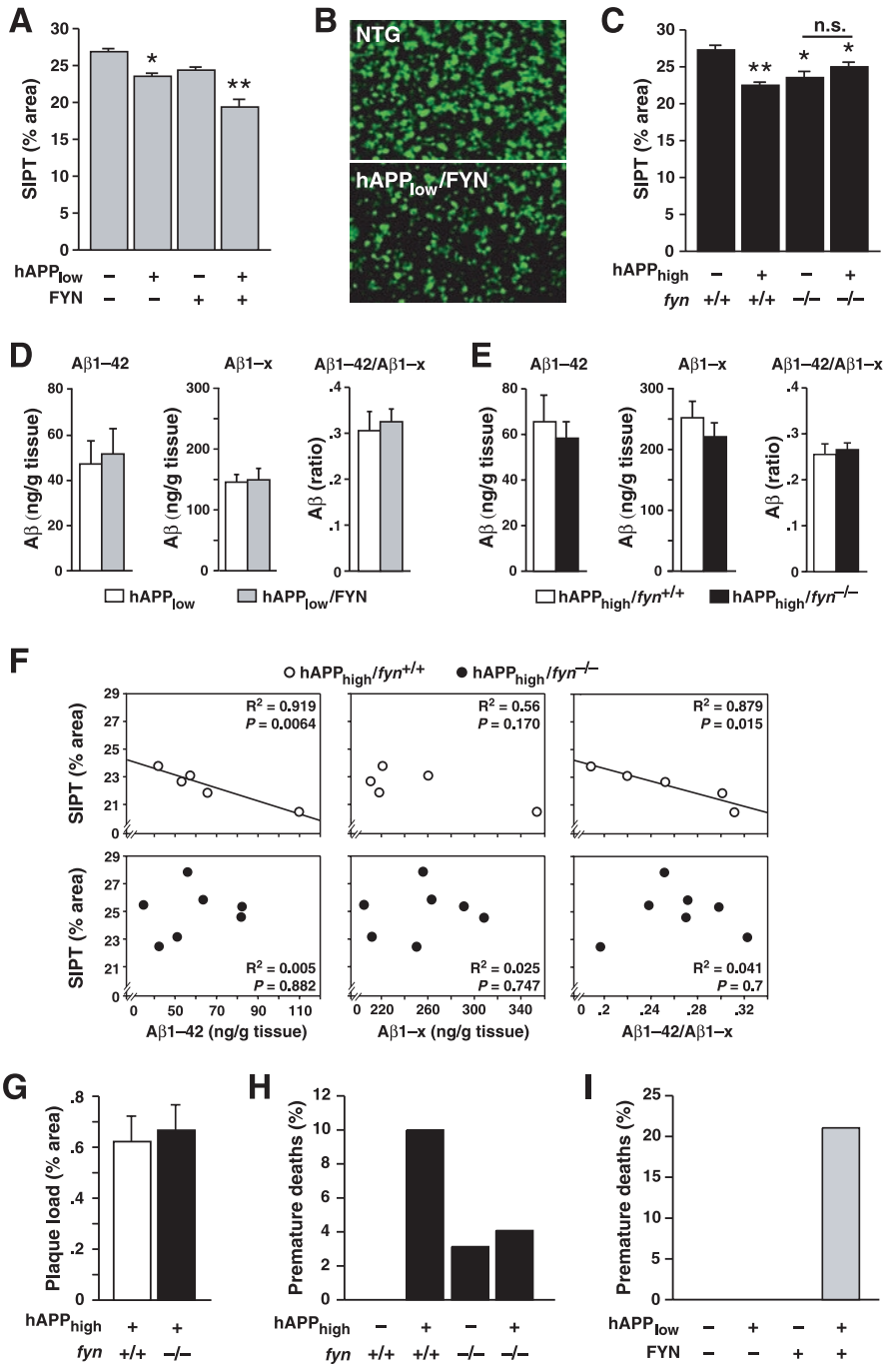

Figure 1. Genetic modulation of mouse Fyn alters the extent of hAPP$_{FAD}$/Aβ-induced reductions in SIPT and premature mortality. FYN refers to the Fyn transgene, and *fyn* refers to the endogenous gene. n.s., Not significant. *$p = 0.001$; **$p < 0.0005$ versus NTG wild-type littermate controls. *A*, Overexpressing Fyn exacerbated SIPT reductions in hAPP$_{low}$ mice ($n = 5$–9 mice/genotype; age, 6–8 months). *B*, Confocal microscopic images illustrating SIPT in the OML of an NTG mouse (top) and an hAPP$_{low}$/FYN doubly TG mouse (bottom). *C*, Ablating Fyn reduced SIPT levels but also prevented additional SIPT reductions in hAPP$_{high}$ mice ($n = 5$–8 mice/genotype; age, 4–5 months). *D*, Overexpressing Fyn did not alter Aβ levels in hAPP$_{low}$ mice ($n = 5$–9 mice/genotype; age, 6–8 months). *E*, Ablating Fyn did not alter Aβ levels in hAPP$_{high}$ mice ($n = 5$–8 mice/genotype; age, 4–5 months). *F*, SIPT levels correlated inversely with Aβ1-42 levels and Aβ1-42/Aβ1-x ratios in hAPP$_{high}$ mice on the *fyn*$^{+/+}$, but not *fyn*$^{-/-}$, background (age, 4–5 months). *G*, Comparable plaque load in hAPP$_{high}$ mice on *fyn*$^{+/+}$ and *fyn*$^{-/-}$ backgrounds ($n = 7$–8 mice/genotype; age, 8–10 months). *H*, *I*, Proportion of mice that died prematurely in each of the groups indicated during the first 6 months postnatally (percentage of 38–64 mice/genotype). Husbandry conditions were similar for all groups.

Discussion

We have identified a mechanistic dichotomy in the cellular effects elicited by hAPP$_{FAD}$/Aβ *in vivo*. hAPP$_{FAD}$/Aβ induces both synaptotoxicity and aberrant growth processes. Our results suggest that only the former involves Fyn kinase-dependent signaling pathways.

A significant correlation between Aβ levels and SIPT levels was observed in hAPP$_{high}$ mice on the *fyn*$^{+/+}$, but not *fyn*$^{-/-}$, background, suggesting that Fyn is necessary for hAPP$_{FAD}$/Aβ to affect SIPT levels. One caveat is that SIPT levels were lower in

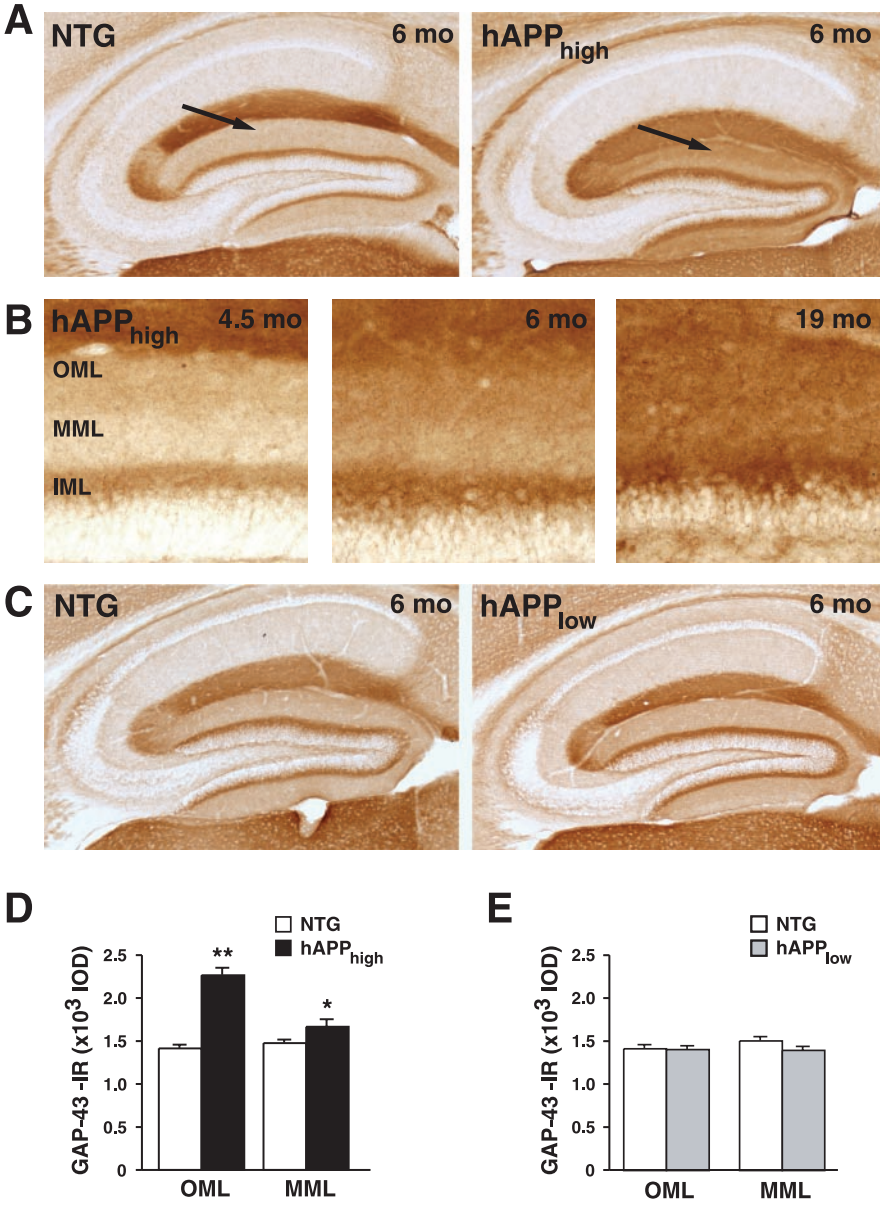

Figure 2. Aberrant axonal sprouting in hAPP$_{FAD}$ mice depends on age and hAPP$_{FAD}$ expression levels. *A*, Photomicrographs of GAP-43-IR in the hippocampus of a 6-month-old NTG mouse and an hAPP$_{high}$ TG littermate. Prominent aberrant sprouting is evident in the molecular layer of the hAPP$_{high}$ mouse (arrow). *B*, In hAPP$_{high}$ mice, GAP-43-IR in this layer further increased with age. *C*, Photomicrographs showing normal GAP-43-IR in the hippocampus of a 6-month-old NTG mouse and an hAPP$_{low}$ TG littermate. *D*, Quantitation of GAP-43-IR in 6-month-old hAPP$_{high}$ mice revealed a 60% increase in the OML and a 13% increase in the MML ($n = 10–13$ mice/genotype). *$p < 0.05$; **$p < 0.0001$ versus NTG wild-type littermates. *E*, Quantitation of GAP-43-IR in the OML and MML revealed no aberrant sprouting in hAPP$_{low}$ mice ($n = 11–13$ mice/genotype). mo, Months; IML, inner molecular layer.

fyn$^{-/-}$ mice than in *fyn*$^{+/+}$ mice even in the absence of hAPP$_{FAD}$/Aβ expression, possibly relating to other hippocampal abnormalities (Grant et al., 1992; Kojima et al., 1997), which could create a "floor" effect in *fyn*$^{-/-}$ mice, preventing additional reductions by other insults. However, increased expression of Fyn exacerbated SIPT reductions in hAPP$_{low}$ mice, providing additional direct evidence for a copathogenic role of Fyn in hAPP$_{FAD}$/Aβ-induced synaptotoxicity.

Increased expression of Fyn by itself did not increase premature mortality in singly TG FYN mice, consistent with previous findings (Kojima et al., 1998). However, ablating Fyn decreased, whereas overexpressing Fyn increased premature mortality in hAPP$_{FAD}$ mice, suggesting that Fyn plays a key role in this disease manifestation. Because overexpression of Fyn was restricted to forebrain neurons (Kojima et al., 1998), our findings suggest a central mechanism. Although we did not witness seizures or the premature deaths during this study, we cannot exclude the involvement of epileptiform activities. hAPP mice on an FVB/N background were more prone to seizures and premature mortality than NTG littermates (Hsiao et al., 1995). Furthermore, Fyn phosphorylates the subunit 2B of the NMDA receptor, and FYN TG mice showed accelerated kindling, which was retarded by NMDA receptor antagonists (Kojima et al., 1998). Thus, increased expression of Fyn may lower the seizure threshold in hAPP$_{FAD}$ mice.

In contrast to synaptotoxicity and premature mortality, axonal sprouting was not altered by genetic modulation of Fyn in hAPP$_{FAD}$ mice. Aberrant sprouting is prominent in AD (Geddes et al., 1985; Masliah et al., 1991; Arendt, 2001) and has also been demonstrated in other lines of hAPP$_{FAD}$ mice (Phinney et al., 1999; Bronfman et al., 2000; Jaffar et al., 2001). hAPP$_{high}$ mice exhibited sprouting on both *fyn*$^{+/+}$ and *fyn*$^{-/-}$ backgrounds, and the magnitude of sprouting increased with age. In contrast, no sprouting was observed in hAPP$_{low}$ mice, even when Fyn was overexpressed. Thus, aberrant sprouting in hAPP$_{FAD}$ mice depends on hAPP$_{FAD}$/Aβ levels and age but not on Fyn.

It is possible that Fyn-dependent synaptotoxicity is caused by Aβ, whereas Fyn-independent aberrant sprouting is caused by an alternative effect of the FAD mutation, another APP metabolite, or the precursor molecule itself. Consistent with this interpretation, SIPT reductions correlated with Aβ levels on the *fyn*$^{+/+}$, but not *fyn*$^{-/-}$, background, and SIPT reductions were worsened by overexpression of Fyn, whereas axonal sprouting did not correlate with Aβ levels on either background and was not worsened by overexpression of Fyn.

Alternatively, synaptotoxicity, premature death, and aberrant sprouting may all be caused by Aβ, but through mechanistically distinct pathways that do or do not involve Fyn. The lack of correlation between aberrant sprouting in the dentate gyrus and Aβ levels does not necessarily invalidate this interpretation because the sprouting stimulus could act on neurons in other regions that project to the molecular layer of the dentate gyrus. In that case, sprouting might correlate with Aβ levels in regions containing the cell bodies of the projections, a possibility that remains to be tested.

Although additional research is needed to define the precise interactions between Aβ and Fyn-related signaling pathways, there is evidence that integrins, cadherins, *N*-syndecan, FAK, paxillin, and tau-phosphorylating kinases might be involved

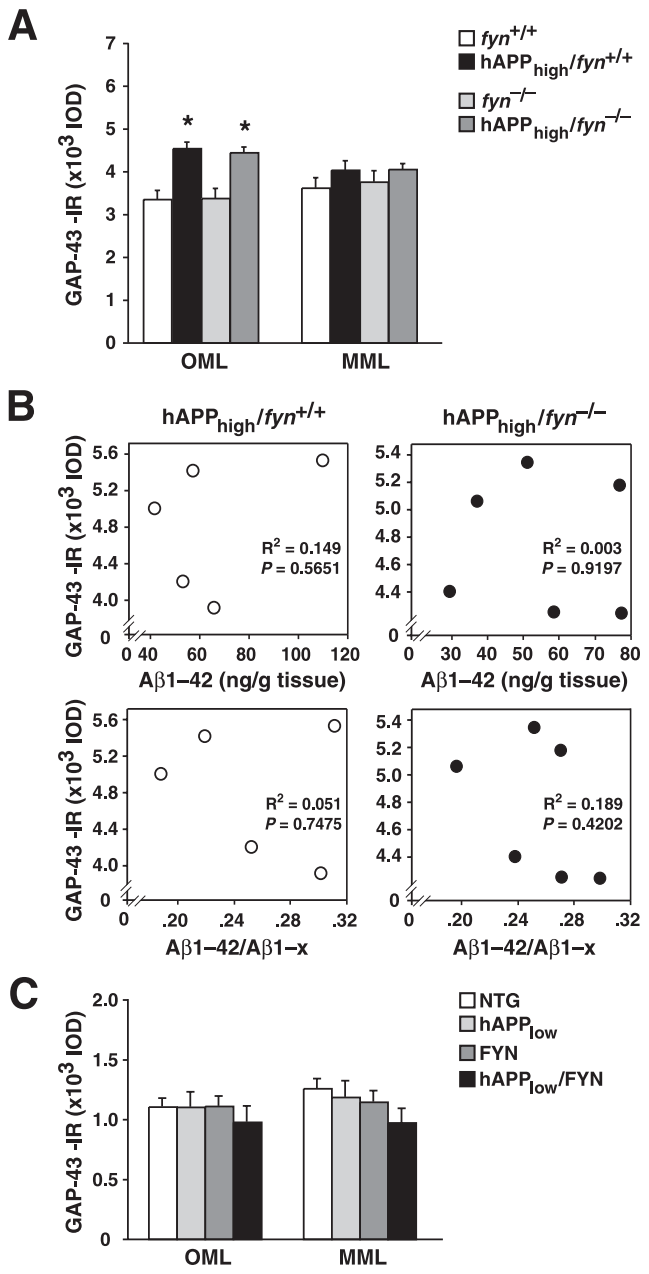

Figure 3. Genetic modulation of Fyn does not affect hAPP/Aβ-induced aberrant sprouting. *A*, Comparable increases in GAP-43-IR in the OML of hAPP$_{high}$ mice on the *fyn* $^{+/+}$ and *fyn* $^{-/-}$ background ($n = 5–8$ mice/genotype; age, 4–5 months). *$p < 0.005$. *B*, GAP-43-IR did not correlate with hippocampal Aβ1-42 levels or Aβ1-42/Aβ1-x ratios in hAPP$_{high}$ mice on the *fyn* $^{+/+}$ and *fyn* $^{-/-}$ backgrounds. *C*, Overexpressing Fyn did not increase GAP-43-IR levels in hAPP$_{low}$ mice ($n = 5–9$ mice/genotype; age, 6–8 months).

(Shirazi and Wood, 1993; Kohmura et al., 1998; Lauri et al., 1999; Chavis and Westbrook, 2001; Williamson et al., 2002; Grace and Busciglio, 2003).

If Aβ affects Fyn itself, Fyn might be required to mediate some of the biological effects of Aβ. Notably, activation of Fyn can have different consequences in different brain regions. In the hippocampus, but not in the cerebral cortex, Fyn is targeted to the NR2B subunit of the NMDA receptor by the scaffolding protein RACK1 (Yaka et al., 2003). Peptides that disrupt interactions between RACK1, NR2B, and Fyn induce phosphorylation of NR2B and potentiate NMDA receptor-mediated currents (Yaka et al., 2002). Thus, increased Fyn activity might elicit excitotoxic neuronal injury in the hippocampus but not in other regions. If Aβ affects targets downstream of Fyn, Fyn might act primarily to prime or amplify Aβ-induced pathogenic cascades. These possibilities are not mutually exclusive and provide a framework of testable hypotheses.

In conclusion, our study demonstrates an important role of Fyn in hAPP/Aβ-dependent synaptic deficits and premature death. It implies that pharmacological modulation of Fyn or related signaling pathways might be of therapeutic benefit in AD.

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