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APP Processing and Synaptic Function

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Summary

A large body of evidence has implicated Aβ peptides and other derivatives of the amyloid precursor protein (APP) as central to the pathogenesis of Alzheimer's disease (AD). However, the functional relationship of APP and its proteolytic derivatives to neuronal electrophysiology is not known. Here, we show that neuronal activity modulates the formation and secretion of Aβ peptides in hippocampal slice neurons that overexpress APP. In turn, Aβ selectively depresses excitatory synaptic transmission onto neurons that overexpress APP, as well as nearby neurons that do not. This depression depends on NMDA-R activity and can be reversed by blockade of neuronal activity. Synaptic depression from excessive Aβ could contribute to cognitive decline during early AD. In addition, we propose that activity-dependent modulation of endogenous Aβ production may normally participate in a negative feedback that could keep neuronal hyperactivity in check. Disruption of this feedback system could contribute to disease progression in AD.

Introduction

Alzheimer's disease (AD), the most common form of dementia in the elderly, is a progressive neurodegenerative disorder that is pathologically characterized by extracellular deposits of β amyloid (Aβ) in senile plaques, intraneuronal neurofibrillary tangles, depressed brain function, and neuronal death (reviewed in Price and Sisodia, 1998). It is now widely believed that the accumulation of Aβ, a small peptide with a high propensity to form aggregates, is central to the pathogenesis of disease (Selkoe, 2000). Although the potential neurotoxic properties of Aβ have been known for over a decade (Yankner et al., 1989), it is still not known how Aβ participates in a pathologic cascade resulting in progressive cognitive decline. It is also not known if Aβ, which is detected in both cerebrospinal fluid and plasma in healthy individuals throughout life (Seubert et al., 1992), plays a role in normal physiology.

The proteolytic processing pathways leading to the formation of Aβ from the amyloid precursor protein (APP), a type I membrane protein, have been well characterized in a number of cell lines (Figure 1A) (Selkoe, 2000). APP is delivered to the surface membrane where it is subject to proteolytic processing by α-secretase. APP molecules that fail to be cleaved by α-secretase can be internalized into endocytic compartments and subsequently cleaved by β-secretase (BACE) and γ-secretase to generate Aβ. A fraction of Aβ peptides are also generated in the Golgi apparatus and, to a lesser extent, the endoplasmic reticulum. Aβ peptides generated in the Golgi and in recycling compartments can be secreted into the extracellular space (Greenfield et al., 1999). The majority of secreted Aβ peptides are 40 amino acids in length (Aβ40), although the smaller fraction of longer, 42 amino acid species (Aβ42) have received greater attention due to the propensity of these peptides to nucleate and drive production of amyloid fibrils (Jarrett et al., 1993).

Recent lines of experimental evidence have suggested that excessive amounts of Aβ are deleterious to neuronal function, in addition to, or in lieu of, its proposed neurotoxic effects. First, addition of Aβ in various aggregation states to neuronal preparations has been shown to elicit electrophysiological phenotypes (Cullen et al., 1997; Freir et al., 2001; Hartley et al., 1999; Kim et al., 2001; Stephan et al., 2001). However, these studies have been challenged because of the difficulties in working with the peptides, whose biological properties can depend on aggregation states and peptide size and composition (Fezoui et al., 2000; Walsh et al., 1999). Furthermore, the relevant subcellular sites and (patho)-physiological concentrations are not known and thus difficult to mimic by exogenous application. Alternative approaches using transgenic mouse models expressing various naturally occurring familial AD-linked mutants of APP have also yielded valuable insights into the physiological and behavioral consequences of excessive Aβ production and accumulation (Chapman et al., 1999; Fitzjohn et al., 2001; Hsia et al., 1999; Larson et al., 1999; Westerman et al., 2002). However, the interpretations of these studies are complicated by the fact that the transgenes are expressed at high levels throughout development and aging. Furthermore, several domains

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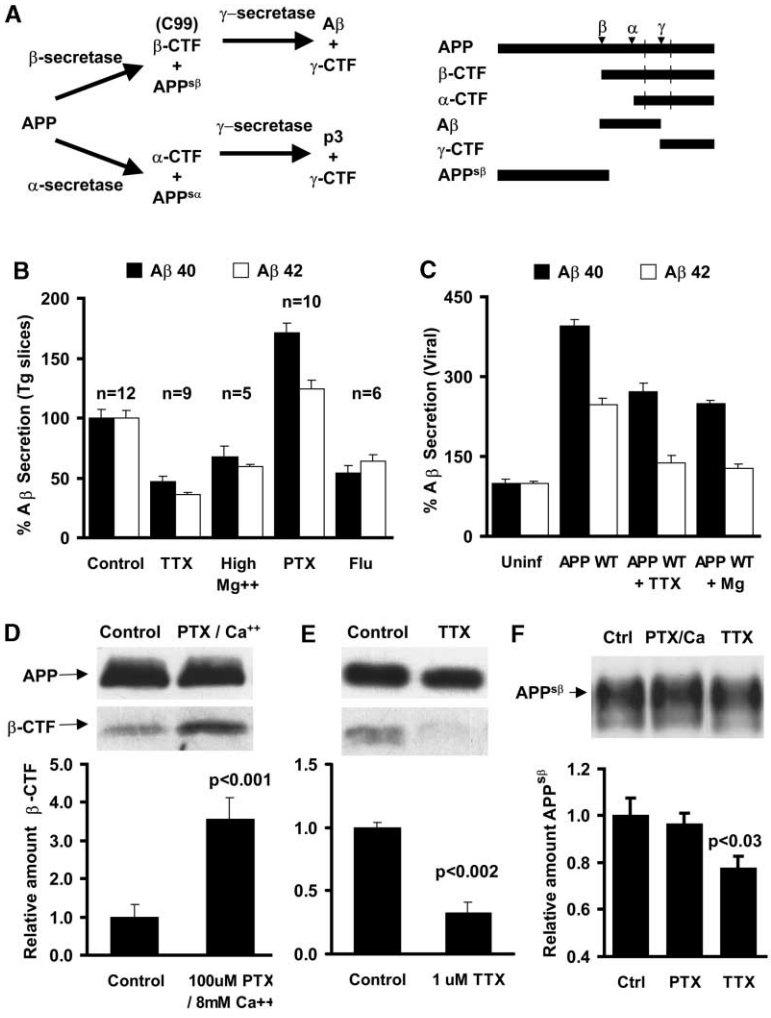

Figure 1. Neural Activity Controls Formation of APP Cleavage Derivatives

(A) Biochemical pathways leading to the formation of Aβ from APP. α and β cleavage of APP result in the production of a large, soluble ectodomain (APP^s) and a membrane-associated carboxy-terminal fragment (CTF). Cleavage of APP by α-secretase precludes production of Aβ. γ-secretase cleavage of CTFs produce small peptides (Aβ and p3) which can be secreted and a truncated CTF (γ-CTF).

(B) Organotypic hippocampal slices prepared from mice expressing human APP_{Swe} were maintained in culture with indicated drugs (tetrodoxin [TTX], 1 μM; 10 mM $MgCl_2$; picrotoxin [PTX], 100 μM; flunitrazepam [Flu], 1 μM). Media aliquots were collected and analyzed for Aβ40 and Aβ42. Values expressed as percentage of secretion seen in sister slices maintained in control media. All values different from control, $p < 0.05$.

(C) Rat organotypic slices were infected (APP WT, APP WT + TTX, APP WT + Mg) or not infected (Uninf) and maintained in culture media (Uninf, APP WT), in culture media with 1 uM TTX (APP WT + TTX), or in culture media with 10 mM MgCl (APP WT + Mg) for 24 hr (n = 3 for each). Superfusate media was collected and analyzed for Aβ40 or Aβ42 as above.

(D) Western blots of extracts from organotypic APP_{SWE} slices in the presence of γ-secretase inhibitor 1 μM L-685,458 (to prevent degradation of β secretase products and therefore aiding fragment detection) treated with (n = 8) or without (n = 8) picrotoxin (100 μM) and 8 mM $CaCl_2$ for 36 hr. Blots were probed with an antibody specific for +1 BACE cleavage site (3D6) and subsequently reprobed using CT-15 for detection of full-length APP. Bands corresponding to the BACE APP cleavage product and full-length APP were quantified by scanning.

(E) Western blots of extracts form organotypic APP_{SWE} slices in the presence of L-685,458 and treated with (n = 3) or without (n = 3) TTX for 36 hr as in (D). Note that levels of full-length APP are not significantly changed by either blocking or enhancing neuronal activity (control: 1.0 ± 0.13, TTX: 1.0 ± 0.04, p = 0.9; control: 1.0 ± 0.11, PTX/Ca^{2+}: 1.2 ± 0.15, p = 0.3).

(F) IP-Western blot of culture media from organotypic APP_{SWE} slices treated for 36 hr with 1 μm TTX (n = 8), picrotoxin (100 mm) and 8 mM $CaCl_2$ (n = 8), or untreated (n = 8). $APP_{SWE}{}^{s\beta}$ was precipitated from culture media using antibody 54, which specifically recognizes the C terminus of $APP_{SWE}{}^{s\beta}$, and subsequently blotted with the antibody 22C11, which recognizes the N terminus domain of APP. 1 μm L-685,458 was present in all samples to parallel (D) and (E) above.

within APP have been described that may have important physiological functions (Cao and Sudhof, 2001; Kamal et al., 2001), and thus, the consequence of overexpressing APP may have unanticipated effects on physiological and behavioral measures of learning and memory. We thus sought to develop a system where the physiologic effects of acute APP overexpression could be ascertained and in which the relevant molecular determinants could be dissected. We employed the Sindbis expression system (Hayashi et al., 2000; Schlesinger and Dubensky, 1999; Shi et al., 1999; Zhu et al., 2000) to acutely deliver APP and APP-related constructs in organotypic hippocampal slices over a period of between 12 and 72 hr (Stoppini et al., 1991). In this experimental setting, we could determine the effects of APP overexpression on synaptic transmission, and the effects of synaptic transmission on APP processing.

The regulatory mechanisms that control Aβ biosynthesis have received much interest, as these are attractive targets for therapeutic intervention. While a number of proteins have been identified whose expression appear to influence Aβ production (Sabo et al., 2001; Sastre et al., 1998; Yu et al., 2000), there is little information about the neuronal mechanisms that modulate Aβ production. Here, we show that neuronal activity regulates the production and secretion of Aβ by controlling APP processing upstream of γ-secretase activity. In addition, we report that Aβ modulates synaptic strength. Taken together, these two observations suggest a negative feedback role for Aβ, which can be revealed by inhibition

of γ-secretase activity. Thus, Aβ may play a role in normal synaptic physiology, as well as in pathological processes leading to AD.

Results

Neuronal Activity Controls Formation and Secretion of Aβ

To examine Aβ secretion in neuronal tissue, we measured (Suzuki et al., 1994) Aβ in the media collected from organotypic hippocampal slices prepared from transgenic (tg) mice (Borchelt et al., 1997) expressing the Swedish APP mutation (APP_{SWE}). This mutation causes autosomal dominant familial AD in two Swedish pedigrees and has been shown to enhance production of Aβ peptides (Citron et al., 1992). To determine the effects of neuronal activity on Aβ secretion, slices were maintained in the presence of pharmacological agents that either decrease (tetrodotoxin, high magnesium, or flunitrazepam [a GABA-A receptor potentiator]) or increase (picrotoxin [a GABA-A channel blocker]) neuronal activity. Agents that decreased or increased activity resulted in significant reductions or elevations, respectively, in levels of Aβ (both Aβ40 and Aβ42) detected in the medium (Figure 1B). These results indicate that the secretion of Aβ from neuronal cells that chronically overexpress APP can be controlled by neuronal activity.

We next wished to evaluate if neuronal activity regulates Aβ secretion by controlling β- or γ-secretase cleavage. These two possibilities can be distinguished by examining the level of β-CTF, the membrane-tethered fragment of APP generated by the action of β-secretase (BACE), that is also a substrate for γ-secretase (Figure 1A). We reasoned that if neuronal activity enhances only γ-secretase cleavage, then β-CTF levels should decrease. On the other hand, if β-secretase cleavage alone is enhanced by neuronal activity, then β-CTF levels should increase. Lastly, if both γ- and β-secretase activities are enhanced by increased neuronal activity, then β-CTF levels should not change significantly. To address this issue, we conducted Western blot analysis on lysates of organotypic slices prepared from APP_{SWE} tg mice. We probed blots with an antibody raised against the carboxy-terminal 15 amino acids of APP that recognizes full-length APP (CT-15), or an antibody that specifically recognizes the amino terminus of β-CTF (3D6). As shown in Figure 1D, increasing neuronal activity by incubating slices in picrotoxin and elevated Ca^{2+} significantly enhanced the levels of β-CTF. Furthermore, decreasing neuronal activity by incubating slices in TTX significantly decreased levels of β-CTF (Figure 1E). In addition, we examined the effects of neuronal activity on the levels of secreted $APPs^{\beta}$, which should parallel the changes in β-CTF, if APP processing is altered. As expected, incubating slices in TTX reduced the amount of $APPs^{\beta}$ released into the culture medium. The levels of $APPs^{\beta}$ were not increased by incubating slices in PTX and high Ca^{2+}, suggesting that activity may also affect degradation of $APPs^{\beta}$. Together, these results suggest that the level of BACE cleavage can be controlled by neuronal activity. Our experiments, however, do not establish if neuronal activity affects intrinsic BACE activity, or if neuronal activity affects the accessibility of APP to BACE activity.

To test if acutely overexpressed APP is under similar regulation, we used a Sindbis expression system to deliver APP to rat organotypic slice neurons. Media from slices expressing recombinant APP for 24 hr released more Aβ (both Aβ40 and Aβ42) into the medium compared to uninfected slices. This secretion could be reduced by inhibiting neuronal activity during the incubation period with TTX or high Mg^{2+} (Figure 1C), indicating that production and secretion of Aβ from neurons that acutely overexpress APP is under similar activity-dependent regulation as that seen in neurons that express transgene-derived APP_{SWE}. Lastly, we also found that the low-level secretion of endogenous Aβ from rat organotypic slices could also be reduced by incubation with TTX for 24 hr (Aβ40: control: 410.5 ± 36.4 pM [$n = 4$], TTX: 274.0 ± 27.3 pM [$n = 3$], $p < 0.03$; Aβ42: control: 39.4 ± 2.4 pM [$n = 4$], TTX: 25.8 ± 3.3 pM [$n = 3$], $p < 0.03$), suggesting that endogenous Aβ secretion operates under a similar regulatory mechanism.

APP Overexpression Depresses Synaptic Transmission

To address whether APP can control synaptic function, we overexpressed APP or various APP mutants in wild-type rat hippocampal slice neurons using the Sindbis expression system. For these experiments, neurons were sparsely infected. Infected neurons were identified by coexpressing (cytoplasmic) diffusable GFP (see Experimental Procedures). One day after infection, the recombinant APP distributed homogenously throughout the dendritic tree including dendritic spines (the sites of excitatory contacts), with no obvious effects on dendritic morphology (Figure 2A). We tested the effects of APP overexpression on synaptic transmission by comparing synaptic responses evoked onto side-by-side pairs of simultaneously recorded postsynaptic neurons where only one neuron expresses the exogenous protein. Excitatory synaptic responses onto neurons expressing recombinant APP were significantly depressed one day after expression, while inhibitory (GABA) currents were unaffected (Figure 2B) (see Experimental Procedures for details of response isolation and measurement). No such depression occurred onto cells expressing only GFP (AMPA: control: 100% ± 7.0%, infected: 96% ± 6.1%, $n = 52$, $p = 0.3$; NMDA: control: 100% ± 9.5%, infected: 90 ± 10.4, $n = 52$, $p = 0.2$ [Hayashi et al., 2000]). Neurons expressing APP showed significant decrease in the frequency of miniature EPSCs, with no change in their amplitude (Figure 2C) nor in paired-pulse facilitation (Figure 2D). These results suggest that the depressive effects of APP overexpression are due to a decrease in the number of functional synapses. This could be due to pre- and/or postsynaptic modification(s).

To determine if BACE processing of recombinant APP is required for the depressive effects of APP overexpression, we expressed a mutant form of APP, APP_{MV} (Citron et al., 1995), which shows little cleavage by BACE at the +1 position of Aβ (cleavage at this site is required to generate Aβ40 or Aβ42). Expression of this mutant

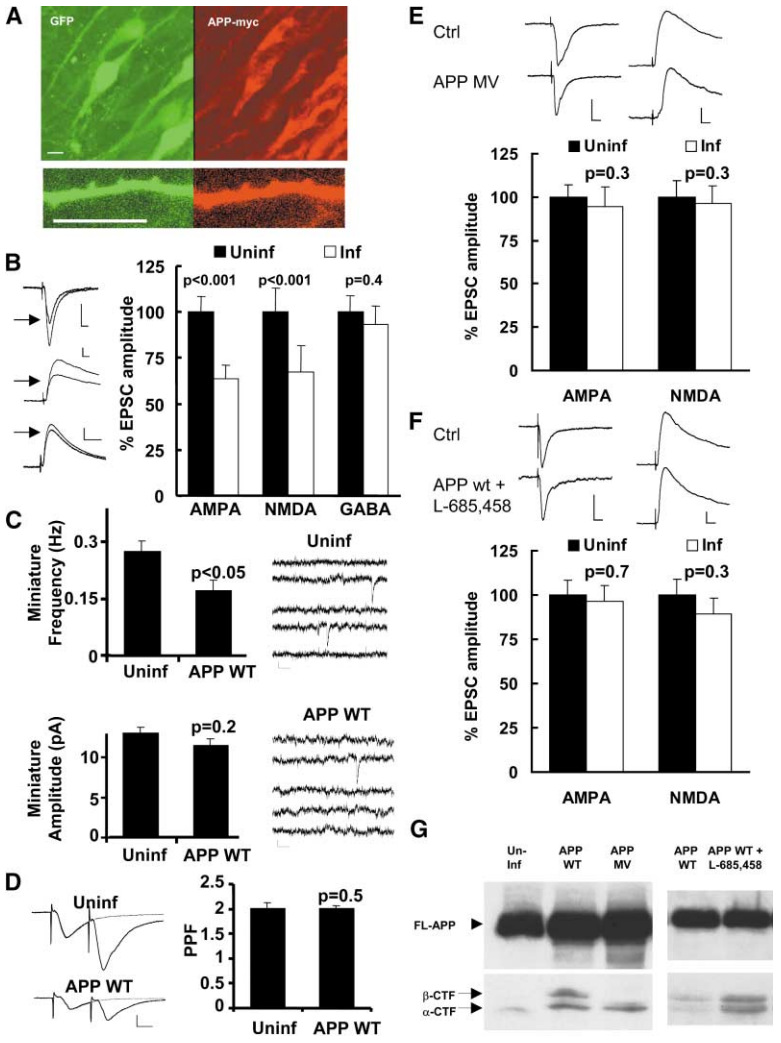

Figure 2. APP Overexpression Depresses Excitatory Synaptic Transmission: Requirements for APP Processing

(A) Two-photon laser scanning microscopic image of organotypic hippocampal slice neurons infected with Sindbis virus expressing APP(myc)-IRES-GFP. Slices were fixed, permeabilized, and immunostained for myc. Left, GFP fluorescence; right, anti-myc fluorescence. Scale bars, 10 μm. Note dendritic spines in lower panels contain recombinant APP.

(B) Graph of AMPA (n = 29), NMDA (n = 25), and GABA (n = 44) components of synaptic transmission measured in pairs of neurons noninfected (filled bars) and infected (open bars) with virus expressing wild-type APP. Inset: sample traces of AMPA (top), NMDA (middle), and GABA (bottom) responses in noninfected and infected (arrow) cells. Scale bars for this and subsequent evoked whole-cell traces: 20 pA, 20 ms.

(C) Miniature EPSC responses recorded in whole-cell mode from neurons in organotypic slices that did not (control, n = 12) or did express APP (n = 10). Frequency of events was diminished (control: 0.27 ± 0.05 Hz, infected: 0.17 ± 0.02 Hz), with no change in their mean amplitude (control: 13.2 ± 0.9 pA, infected: 11.6 ± 0.7 pA) by APP expression. Right, sample traces for each condition (scale bars: 5 pA, 100 ms).

(D) Paired-pulse faciliation evoked onto noninfected (top) and infected (bottom) neurons. Bar graph shows averages from n = 20 cells.

(E) Same graph as (B) for neurons expressing APP_{MV} (AMPA, n = 59; NMDA, n = 45).

(F) Same graph as (B) for transmission recorded from neurons expressing wild-type APP and maintained in the γ-secretase inhibitor L-685,458 (AMPA , n = 29; NMDA, n = 29).

(G) Western blot of hippocampal extracts from slices infected and treated as indicated above. Blots were probed with anti-APP carboxy-terminal antibody, CT-15 (Sisodia et al., 1993).

produced no significant depression of transmission (Figure 2E); the effect on transmission by APP_{MV} was significantly different from the effect of wild-type APP (AMPA: $p < 0.014$ [n = 55, 63]; NMDA: $p < 0.015$ [n = 50, 49], K-S test). Parallel experiments showed that slices infected with this virus express APP_{MV}, and as expected, the APP-CTF product that would be generated by processing by BACE at +1 site is not apparent (Figure 2G). This result indicates that BACE cleavage at the +1 site is necessary for the depressive effects of APP overexpression on transmission. Furthermore, these results indicate that α-secretase-generated α-CTF is unlikely to be responsible for the depressive effects, since this product is still generated from APP_{MV} (Figure 2G).

To assess the requirement for γ-secretase processing of APP on the depressive effect on synaptic transmission, we employed a highly potent and selective γ-secretase inhibitor, L-685,458 (Li et al., 2000). Slices were infected with a virus producing APP and maintained in the presence of L-685,458 for 24 hr. Transmission onto neurons expressing APP was not depressed relative to nearby control cells (Figure 2F). Parallel experiments showed that slices expressing APP in the presence of L-685,458 displayed the expected accumulation of β-CTF and α-CTF, the immediate substrates for γ-secretase (Figure 2G). These studies indicate that APP synthesis and proteolytic steps prior to γ-secretase cleavage were not impaired by the addition of L-685,458. Further, these results indicate that γ-secretase processing of APP is required for the depressive effects of APP and that this phenotype is independent of the formation of the large ectodomain of APP following either α or β cleavage events (Furukawa et al., 1996).

Aβ Production from APP Mediates Depression of Transmission

The requirement for BACE and γ-secretase processing of recombinant APP strongly suggests that Aβ mediates the observed phenotype on neuronal transmission. On the other hand, another carboxy-terminal derivative of APP, termed γ-CTF, or AICD (see Figure 1A) has been described that may serve as a transcriptional coactiva-

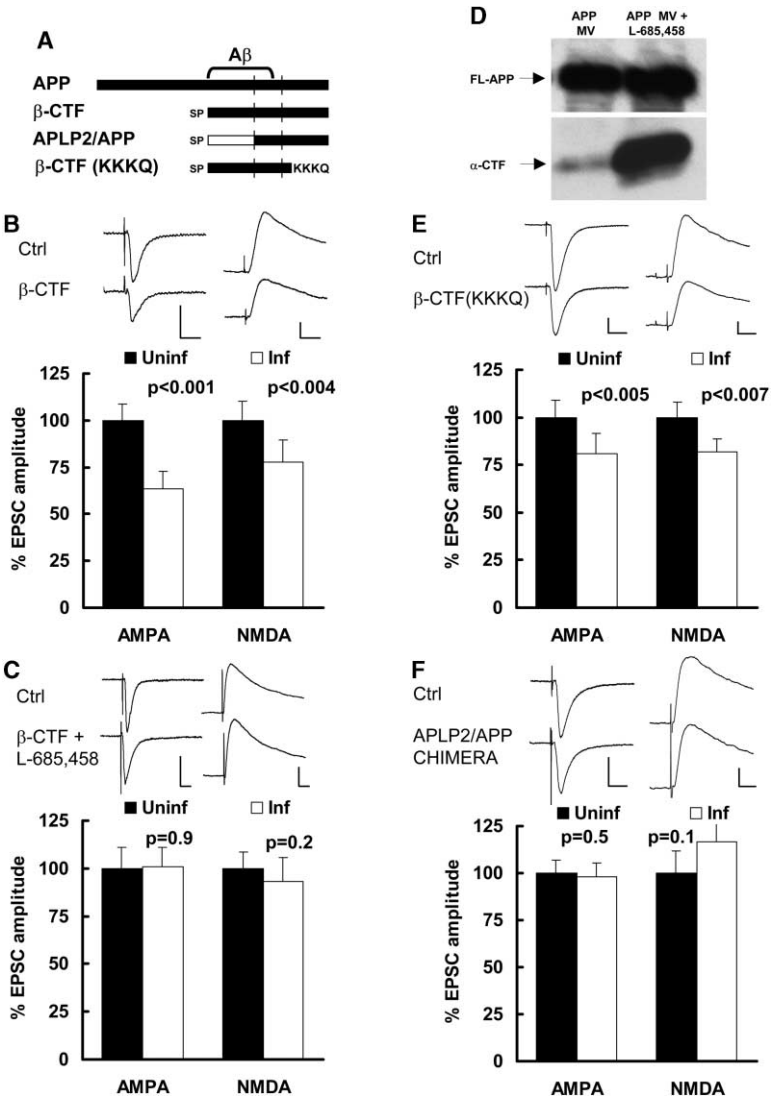

Figure 3. Aβ Domain of APP Is Necessary and Sufficient to Depress Synaptic Transmission

(A) Schematic diagram of APP constructs used in this figure. SP indicates signal peptide.

(B) Graph of AMPA (n = 33) and NMDA (n = 31) components of synaptic transmission measured in pairs of neurons noninfected (filled bars) and infected (open bars) with virus expressing β-CTF. Inset: sample traces of transmission measured at −60mV (left) and +40mV (right) from noninfected (top) and infected (bottom) neurons.

(C) Same as (B) but slices incubated in the presence of L-685,458 (AMPA, n = 40; NMDA, n = 33).

(D) Western blot of hippocampal extracts from slices infected with APP_{MV} in the absence (left) or presence (right) of γ-secretase inhibitor L-685,458. Blots were probed with CT-15 (see Experimental Procedures and Figure 1).

(E) Same as (B) for neurons expressing β-CTF(KKKQ) (AMPA, n = 40; NMDA, n = 32).

(F) Same as (B) for neurons expressing APLP2/APP Chimera (AMPA, n = 35; NMDA, n = 33).

tor (Cao and Sudhof, 2001). However, α-CTF accumulates in cells treated with L-685,458 (Figure 3D), indicating that this APP derivative is a substrate of γ-secretase, and thus likely a precursor of γ-CTF. Taken together with the finding that abundant amounts of α-CTF are produced in APP_{MV}-infected cells (Figure 2G), which show no synaptic depression (Figure 2E), we feel it unlikely that γ-CTF is responsible for the depressive effects on synaptic transmission.

To test more directly whether Aβ is necessary and sufficient to produce a depression of synaptic transmission, we infected hippocampal slice neurons with Sindbis virus harboring cDNA encoding β-CTF, a polypeptide that includes Aβ, and the entire transmembrane and cytoplasmic domains of APP (Figure 3A) [Iwata et al., 2001]). As expected, expression of β-CTF was sufficient to depress synaptic transmission (Figure 3B), and this depression was prevented by incubating slices in the γ-secretase inhibitor L-685,458 (Figure 3C). Indeed, expression of a truncated β-CTF (β-CTFKKKQ [Iwata et al., 2001]; Figure 3A) that contains Aβ, the entire transmembrane domain, and a KKKQ membrane-anchoring motif, but lacks the intracellular C terminus, was able to produce a depression of synaptic transmission (Figure 3E), although not to the extent produced by expression of β-CTF (Figure 3B). The reduced depression by β-CTFKKKQ may be due to inefficient trafficking of this polypeptide to sites of action, since cellular targeting of APP is largely controlled by the APP C terminus (Perez et al., 1999). In any event, these results argue that the Aβ domain is sufficient to depress transmission. As a further test that Aβ is necessary for depression of synaptic transmission, we generated a chimeric β-CTF construct in which the Aβ region was replaced by the corresponding region in APLP2, an APP homolog that has considerable homology with APP throughout most of its length except in the Aβ region, where there is considerable divergence (Wasco et al., 1993) (see Figure 3A). Expression of this APLP2/APP chimera did not depress synaptic transmission (Figure 3F), further supporting the view that the Aβ domain of APP is crucial for the observed synaptic depression. Taken together, these re-

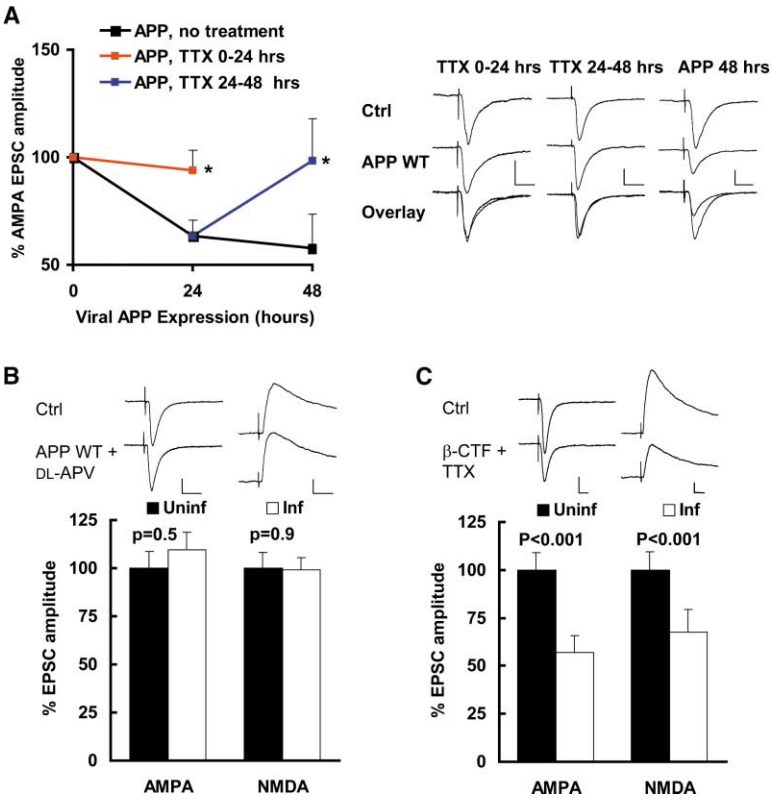

Figure 4. Synaptic Depression Mediated by APP Overexpression Is Activity Dependent and Reversible

(A) Amplitude of AMPA-mediated synaptic transmission recorded in neurons expressing APP WT for 24 or 48 hr (n = 27). Slices were maintained in normal medium (black), in TTX for hours 0–24 (red, n = 23), or in TTX for hours 24–48 (blue, n = 37). * indicates significant difference from value obtained at same time point in different conditions. Inset: sample traces of transmission measured at −60mV for each of the time points.

(B) Graph of AMPA (n = 33) and NMDA (n = 32) components of synaptic transmission measured in pairs of neurons noninfected (filled bars) and infected (open bars) with virus expressing APP WT incubated in the presence of 100 uM D,L AP5. Inset: sample traces of transmission measured at −60mV (left) and +40mV (right) from noninfected (top) and infected (bottom) neurons.

(C) Same as (B) for neurons expressing β-CTF and maintained in TTX (AMPA, n = 36; NMDA, n = 32).

sults indicate that the Aβ domain is necessary and sufficient for APP overexpression to produce synaptic depression.

APP-Induced Depression Requires Neuronal Activity

The results shown above indicate that processing of APP into Aβ is dependent on neuronal activity and that formation of Aβ results in synaptic depression. Thus, a direct prediction of these findings is that blockade of neuronal activity should prevent the depressive actions of APP overexpression. To test this prediction, we maintained slices in conditions where spontaneous neuronal activity was blocked during the expression of full-length APP and subsequently assayed the effects on physiology. AMPA-mediated synaptic transmission onto neurons that express recombinant APP and maintained in TTX showed no depression of synaptic transmission compared to neurons expressing recombinant APP and maintained in normal medium (Figure 4A; 24 hr with or without TTX, $p < 0.006$, K-S test). Similar results were obtained by measuring NMDA responses (data not shown). It is notable that in conditions where neuronal activity is suppressed, APP-overexpressing neurons demonstrate normal synaptic transmission, near baseline levels of Aβ42, but still significant (albeit reduced) levels of Aβ40 (Figure 1C). This suggests that Aβ42, rather than Aβ40, may more potently contribute to the observed synaptic depression.

We tested the generality of this activity-dependent effect by using other agents known to suppress neuronal activity. Indeed, 10 μM NBQX (an AMPA receptor antagonist) added to the culture media during the incubation period also blocked APP-induced depression (AMPA: control: 100% ± 11.3%, infected: 100% ± 12%, n = 28, p = 0.7; NMDA: control: 100% ± 10.7%, infected: 90% ± 9.7%, n = 27, p = 0.3). We next tested whether synaptic NMDA receptor activation could prevent synaptic depression produced by APP overexpression. Slices were incubated with 100 μM D,L AP5, an agent that blocks NMDA-Rs and has previously been shown not to affect spontaneous neuronal activity (Zhu et al., 2000). Notably, blockade of NMDA receptors prevented the depression caused by APP overexpression (Figure 4B). Thus, it appears that NMDA receptor activation by spontaneous neuronal activity is required for APP overexpression to depress synaptic transmission.

Our results above suggest that neural activity affects Aβ production by controlling BACE cleavage of APP. Another prediction from this finding is that blockade of neural activity should not prevent the synaptic depression produced by expression of β-CTF. Indeed, while the γ-secretase inhibitor did block synaptic depression by β-CTF (Figure 3C), TTX did not (Figure 4C). Taken together, these results provide physiological and biochemical support for the notion that neuronal activity promotes production of Aβ by modulating BACE processing of APP.

Depressive Effects of Aβ Are Reversible

To determine if the depressive effects on transmission by APP overexpression are reversible, we initially placed slices overexpressing APP in normal culture media for 24 hr; TTX was then added to fresh media for 24 hr, and synaptic transmission was subsequently assayed. Remarkably, after such a protocol, synaptic responses

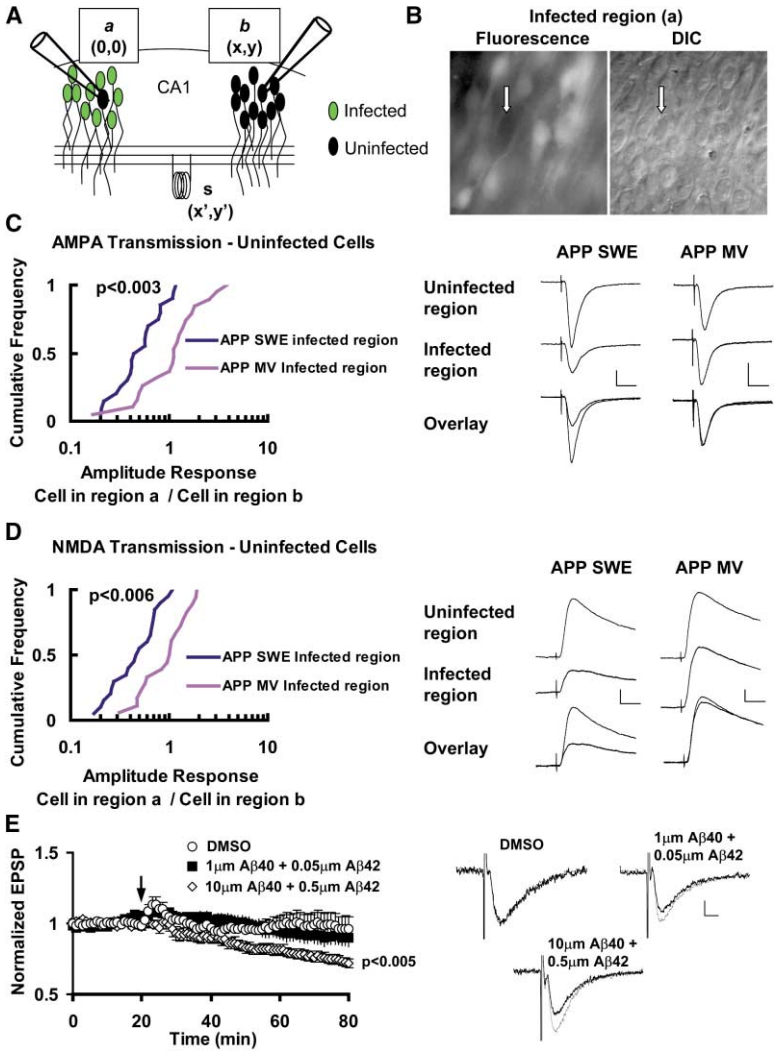

Figure 5. Secreted Aβ Can Depress Transmission onto Nearby Neurons

(A) Schematic of experimental paradigm. Uninfected neuron is surrounded by infected neurons in region a; uninfected neuron is surrounded by uninfected neurons in region b. Either cell *a* or cell *b* was assigned coordinates (0,0) for simplicity; s indicates coordinates of stimulating electrode.

(B) Fluorescent (left) and DIC (right) images of a small region within the CA1 infected with a high titer APP virus. Arrow indicates an uninfected cell surrounded by many infected cells.

(C) Comparison of AMPA transmission between simultaneously recorded pairs of uninfected neurons in the two different regions. Cumulative distributions were computed using the ratio of the amplitude response of a neuron in *region a* to the amplitude response of a neuron in *region b*. Separate distributions were computed for APP_{SWE} and APP_{MV}. Amplitude response ratios are reported in a log scale for display purposes. Inset: sample traces of AMPA transmission for a pair of uninfected neurons, one neuron surrounded by APP_{SWE}-expressing neurons (left), or APP_{MV}-expressing neurons (right).

(D) Same as (C) for NMDA transmission.

(E) Field recordings of evoked EPSPs monitored from acute rat slices exposed to varying amounts of Aβ peptides. After obtaining stable baseline responses for 20 min, freshly dissolved Aβ peptides (1 μm Aβ40 + 0.05 μm Aβ42 or 10 μm Aβ40 + 0.5 μm Aβ42) were added to the circulating ACSF, and responses were monitored for the next 40 min.

in infected cells recovered to control levels (Figure 4A). Expression of APP for 2 days with no TTX treatment showed no such recovery (Figure 4A; difference between 24 hr no drug followed by 24 hr TTX versus 48 hr no drug, $p < 0.004$, K-S). Similar results were obtained for NMDA responses (data not shown). Thus, preventing neuronal activity for 24 hr can reverse the depression of transmission produced by acute APP overexpression. Our experiments, however, do not indicate if the effects of chronic APP overexpression can be reversed.

Aβ Depresses Transmission in a Noncell-Autonomous Manner

We wished to test whether Aβ produced from overexpressing neurons can affect neighboring neurons. The experiments above show that overexpression of APP in a neuron can cause synaptic depression onto that neuron, compared to a control nearby neuron. One interpretation of these results is that Aβ acts only on the cell that produces it, that is, in a strictly cell-autonomous manner. An alternative interpretation is that noninfected, control neurons in these experiments can be affected by the secreted Aβ, but they fail to respond because the amount of Aβ reaching a control neuron is likely to be orders of magnitude lower than the amount secreted by the infected neuron (the expression protocol was purposefully chosen to infect neurons sparsely). To test if secreted Aβ can indeed act *inter*cellularly, we compared synaptic function onto two *un*infected cells (*a* and *b*); cell *a* was chosen from a region containing many infected cells, while cell *b* was chosen from a region with no infected cells (Figures 5A and 5B). The two uninfected cells were selected to be about 500 μM apart, and a stimulating electrode was placed equidistant to both cells (see Experimental Procedures). To maximize Aβ production, we expressed full-length APP_{SWE}. Excitatory synaptic responses were recorded simultaneously from cells *a* and *b*, and a ratio of the amplitude response (response of cell *a*/response of cell *b*) was computed. Uninfected neurons surrounded by APP_{SWE} had similar baseline electrophysiological properties as neurons from uninfected regions (input resistance: uninfected region, 215 ± 15 MOhm ($n = 7$); infected region, 205 ± 12 MOhm [$n=14$]; $p > 0.6$). In accordance with the prediction that Aβ can affect neurons in a noncell-autonomous manner, uninfected neurons surrounded by APP_{SWE}-infected neurons had significantly depressed

transmission when compared to distant control neurons (~50% reduction in both AMPA- [$n = 20$, $p < 0.001$] and NMDA- [$n = 20$, $p < 0.001$] mediated synaptic transmission; Figure 5C and 5D, inset), suggesting that uninfected neurons in infected regions were responding to the local high concentrations of Aβ. In further support of this view, responses from an APP-infected neuron in a heavily infected region were no different from responses onto a nearby noninfected neuron (AMPA: control: 100% ± 7.8%, infected: 94% ± 8.1%, $n = 36$, $p = 0.3$; NMDA: control 100% ± 12.3%, infected 93% ± 13.8%, $n = 33$, $p = 0.2$; compare with Figure 2B).

To control for effects other than increased production of Aβ, we performed a comparable experiment using the APP_{MV} mutant, described above. Uninfected neurons surrounded by APP_{MV}-infected neurons had similar levels of transmission when compared to distant control neurons (as expected, no reduction in either AMPA [$n = 19$, $p = 0.7$] or NMDA [$n = 19$, $p = 0.6$]; Figures 5C and 5D, inset). Importantly, the effect by APP_{SWE} on transmission onto uninfected neurons was significantly different from the effect of APP_{MV} (Figures 5C and 5D). Taken together, these results indicate that Aβ from neurons overexpressing APP can depress synaptic transmission onto nearby neurons when sufficient levels of Aβ are achieved.

As a further test of the depressive effects of secreted Aβ, we bath applied synthetic Aβ (1-40 and 1-42) onto hippocampal slices. While it is not known what local concentrations can be attained by secreted Aβ, nor what oligomeric forms render these peptides active, we noted a concentration-dependent depression of transmission (Figure 5E). This is consistent with the view that secreted Aβ depresses synaptic transmission.

Aβ as a Negative Feedback Regulator of Neuronal Activity

The data presented above indicate that increased neuronal activity promotes the formation of Aβ and that increased Aβ formation depresses synaptic function. While these relationships were obtained primarily with overexpressed human APP, they suggest the existence of a negative feedback process wherein higher levels of neuronal activity may increase the production of Aβ from endogenous APP and lead to synaptic depression that could curtail excessive activity. We therefore designed a series of experiments to examine the effects of different levels of APP expression, and different stimulation conditions, on Aβ-induced synaptic depression.

We first tested the effects of a standard LTP-inducing protocol in the context of APP overexpression. Following a pairing protocol (Figure 6A), neurons from organotypic APP_{SWE} transgenic slices showed a transient potentiation which returned to baseline levels within 25 min of LTP induction; potentiation persisted in control neurons for the duration of the experiment (Figure 6A). These results are consistent with the view that acute enhancement of synaptic activity (e.g., a pairing protocol) can drive the production of Aβ, which subsequently depresses synaptic function and offsets LTP. This view is supported by the finding that when using the same conditions, bath application of L-685,458 (the γ-secretase inhibitor) permitted pairing-induced LTP in neurons from APP_{SWE} slices (Figure 6A).

We next considered under what conditions of electrical activity this regulation would be operative when APP and Aβ are expressed at endogenous levels. As our previous experiments relied on expression of human APP, it was important to determine if rodent Aβ, which differs from human Aβ at 3 amino acids, produced a similar phenotype. This sequence difference has been suggested to be responsible for the enhanced toxic and biologically active properties of the human form of the peptide (Johnstone et al., 1991). Expression of rodent β-CTF in neurons was sufficient to depress synaptic transmission (AMPA: control: 100% ± 6.2%, infected: 64.5% ± 8.0%, [$n = 30$, $p < 0.001$]; NMDA: control 100% ± 13.4%, infected 73.5% ± 12.8%; $n = 23$, $p < 0.006$). Thus, we conclude that like human Aβ, rodent Aβ peptides are biologically active and capable of exerting electrophysiological effects, despite their inability to form amyloid pathology in the rodent brain.

In wild-type animals, acute application of L-685,458 had no effect on basal transmission (45 min after drug, 0.95 ± 0.02 of baseline; 45 min after vehicle, 0.96 ± 0.02; $p = 0.8$), no effect on long-term depression (LTD) (after low-frequency stimulation, vehicle control: 70% ± 2.5% [$n = 25$], L-685,458: 73% ± 2.7% ($n = 26$); $p = 0.4$), nor on single-tetanus LTP (Figure 6B). A pairing protocol (generally considered to be a stronger stimulus than a single tetanus) revealed a small (but not significant) increase in potentiation in the presence of the drug (Figure 6A). This effect became more obvious with repeated tetanic stimulation, which produced significantly more potentiation in the presence than in the absence of L-685,458 (Figure 6C). Furthermore, while acute application of L-685,458 produced no detectable effects on baseline transmission (see above), 24 hr application of the drug increased the frequency of miniature EPSCs in wild-type slices (Figure 6D). These results suggest that strong acute electrical activity or low chronic activity can recruit Aβ-induced synaptic depression in wild-type tissue. The stronger stimuli and longer periods required to detect effects of endogenous Aβ is consistent with the fact that brain tissue from rats or mice contain very low steady-state levels of Aβ peptides derived by processing at the +1 BACE site (De Strooper et al., 1995).

Discussion

Activity-Dependent APP Processing and Aβ Secretion

Our studies have identified a novel regulatory mechanism by which individual neurons or neuronal networks may control Aβ production and secretion. Generation of both Aβ40 and the more fibrillogenic Aβ42 can be controlled by neuronal electrical activity. This occurs in the context of APP overexpression in either transgenic (chronic) or virally (acutely) driven settings and also under endogenous levels of APP. These results complement earlier studies indicating that neuronal electrical activity can modulate generation of APP secretory products in wild-type tissue (Nitsch et al., 1993). Our studies are consistent with the notion that neuronal electrical activity modulates APP processing at the β-secretase site. It is now fairly well established that neural activity can regulate the trafficking of proteins at synaptic sites

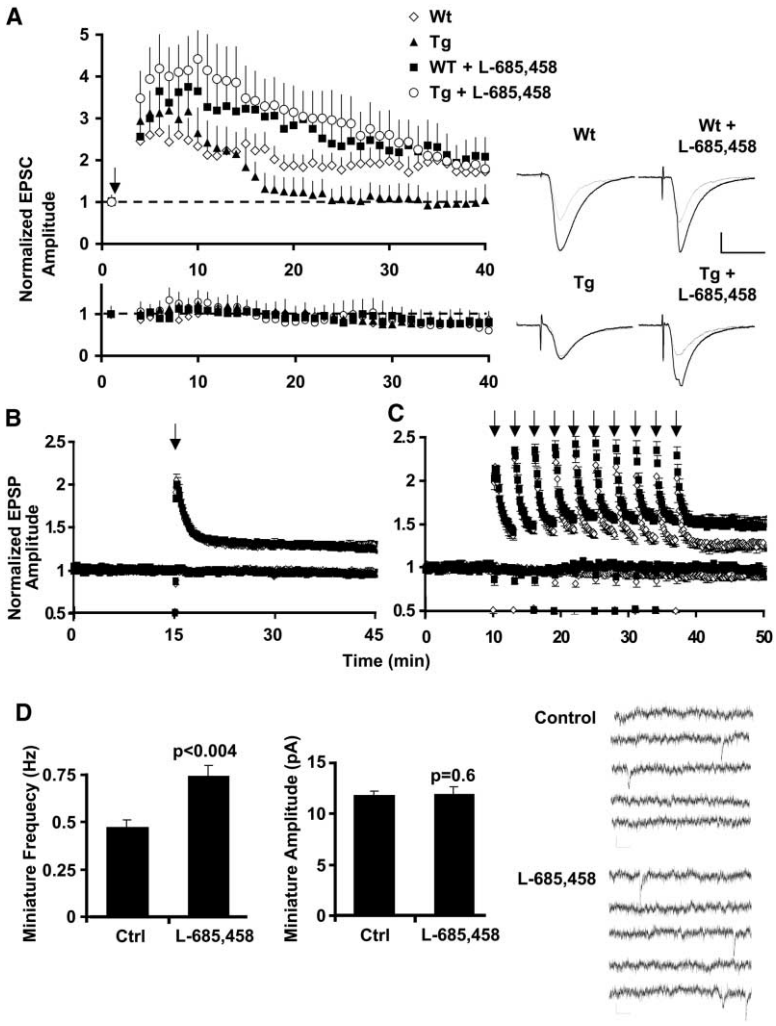

Figure 6. The γ-Secretase Inhibitor L-685,458 Reveals Stimulus Conditions Recruiting Aβ-Induced Depression in Wild-Type Hippocampal Slices

(A) Whole-cell recordings of neurons from WT or APP_{SWE} organotypic mouse slices in the presence or absence of γ-secretase inhibition. After a short baseline, LTP was induced in one pathway by a pairing protocol, while the other pathway was not paired to monitor baseline responses. Evoked AMPA-R-mediated synaptic responses from the paired pathway (top) and control pathways (bottom). Legend: open diamonds: WT slices, DMSO treated; filled triangles: Tg slices, DMSO treated; filled squares: WT slices, L-685,458 treated; open circles: Tg slices, L-685,458 treated. Inset: sample traces of AMPA-R mediated synaptic transmission before (thin) and after (thick) whole-cell pairing protocol.

(B) Field recordings of evoked EPSPs monitored from acute rat slices exposed (open symbols, n = 23) or not exposed (filled symbols, n = 26) to 1 μM L-685,458. At arrow, tetanic stimulation (1 s, 100 Hz) was delivered.

(C) Same as (A) except ten tetanic stimuli were delivered, each tetanus (arrow) separated by 3 min. Responses following ten tetanic stimuli were significantly different, $p < 0.03$ (control n = 17; treated n = 17). Scale bars: 200μV, 10 ms.

(D) Miniature EPSC responses recorded in whole-cell mode from neurons in wild-type organotypic slices maintained in the absence (control, n = 22) or presence of 1 μM L-685,458 (n = 18). Frequency of events was enhanced (control: 0.47 ± 0.03 Hz; L-685,458: 0.75 ± 0.9 Hz) with no change in their mean amplitude (control: 11.8 ± 0.5 pA; L-685,458: 11.9 ± 0.9 pA) by drug treatment. Right, sample traces for each condition.

(e.g., AMPA-Rs [Carroll et al., 2001; Malinow et al., 2000]), and hence, it is possible that neuronal activity promotes the endocytosis of surface APP, enhancing the accessibility of APP to BACE in endosomal/recycling compartments.

It is of interest, and potential clinical relevance, that inhibiting excitatory synaptic transmission or blocking NMDA-Rs prevents the synaptic depressive effects of APP overexpression. Our results are consistent with the findings of two clinical studies. First, the benzodiazepines, agents that enhance inhibitory transmission and thereby decrease excitatory drive, have been found to protect against Alzheimer's disease (Fastbom et al., 1998). This observation is notable in view of our findings that benzodiazepines reduce secretion of Aβ peptides from hippocampal slice neurons (Figure 1B). Second, NMDA receptor antagonists block the APP-induced synaptic depression in our studies, and NMDA-R antagonists have been shown to be effective in slowing cognitive decline in mild to severe AD patients (Reisberg et al., 2000; Winblad and Poritis, 1999).

Aβ Disrupts Neuronal Transmission: a Mechanism for the Early Cognitive Defects of AD?

The mechanisms responsible for the cognitive decline underlying AD are not understood, but it is widely believed that Aβ accumulation in the brain is a critical component. While Aβ can be neurotoxic (Yankner et al., 1990), there is growing evidence that cognitive decline can occur before, or independent of, neuronal loss, as amyloid-dependent physiological and behavioral deficits in transgenic mice can occur in the absence of cell death (Hsiao et al., 1996; Morgan et al., 2000; Westerman et al., 2002). Here, we show that processing of overexpressed APP into Aβ leads to depression of synaptic transmission. This result is consistent with previous studies of transgenic mice expressing APP harboring mutations known to cause early onset familial Alzheimer's disease (Fitzjohn et al., 2001; Hsia et al., 1999). Such mice produce more Aβ and show depressed synaptic function before amyloid plaque deposition becomes evident. Our molecular dissection of the APP molecule reveals that Aβ is responsible for this synaptic depression. Such effects could contribute to the cognitive dysfunction in AD (Selkoe, 2002; Walsh et al., 2002).

A Normal Physiologic Role for Aβ?

While our results are consistent with the notion that high Aβ levels may disrupt synaptic function, our data suggest that Aβ may also have a normal negative feedback function. Increased neuronal activity produces more Aβ; the enhanced Aβ production depresses synap-

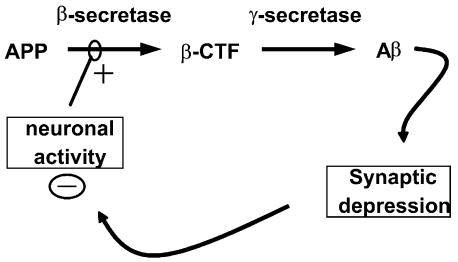

Figure 7. Negative Feedback Model Indicating Proposed Interaction between Neural Activity and APP Processing

Neural activity regulates β-secretase actions on APP. Formation of Aβ depresses synaptic transmission. Synaptic depression decreases neural activity.

tic function; the depressed synaptic function will decrease neuronal activity (Figure 7A) . Examples of synaptic homeostasis have recently been reported (Davis et al., 1998; Turrigiano et al., 1998), as well as intercellular depression following strong tetanic stimulation (Scanziani et al., 1996), although the signaling molecules mediating these processes have not been identified. In support of this model, we find that in addition to human Aβ, rodent Aβ can also depress synaptic transmission. This is important because rodent Aβ is believed not to have amyloidogenic properties. We find that in wild-type rat tissue, multiple tetani lead to greater synaptic potentiation in the presence of the γ-secretase inhibitor L-685,458. This suggests that multiple tetani drive APP processing, producing a synaptic depression (in addition to LTP) that can be revealed by γ-secretase inhibition. This phenomenon is seen when multiple tetani are delivered suggesting that the negative feedback system mediated by APP processing may normally only be rapidly recruited under very high activity levels. This may explain the enhanced kainate-induced seizure activity in APP knockout mice (Steinbach et al., 1998). We also find that 24 hr application of γ-secretase inhibitor L-685,458 leads to enhanced synaptic transmission (increased miniature EPSC frequency, Figure 6D). The absence of an overt phenotype in mice lacking BACE (Cai et al., 2001; Luo et al., 2001) or APP (Zheng et al., 1996) suggests that other mechanisms can compensate for this in these mice.

How could disturbances in this proposed negative feedback loop contribute to AD? One can envision a number of scenarios. For instance, if synapses lose sensitivity to Aβ-induced depression, persistently elevated neuronal activity may go unchecked. High levels of neuronal activity could lead to excitotoxicity (Zoghbi et al., 2000), as well as higher levels of secreted Aβ peptides, which may in turn form neurotoxic fibrils that eventually kill neurons (Cotman et al., 1992; Lambert et al., 1998; Walsh et al., 1999; Yankner et al., 1990). Alternatively, Aβ production may become constitutive (loose sensitivity to synaptic activity), with resulting synaptic depression and neuronal toxicity.

Experimental Procedures

Construction of Plasmids and Pseudoviruses

APP695 (human) and GFP (Clonetech) were coexpressed by using an internal ribosomal entry site (IRES) construct (Hayashi et al., 2000). Full-length APP constructs contain the 12 amino acid myc sequence immediately before the termination codon. APP_{MV} was generated using the Quick Change Mutagenesis Kit (Stratagene). Constructs were cloned into pSinRep5 shuttle vector and infective sindbis pseudoviruses were generated as described (Malinow et al., 1999).

Hippocampal Slices

Organotypic hippocampal slices were prepared from 6-day-old rat or APP_{SWE} transgenic mice (Borchelt et al., 1996) and maintained using standard methods (Stoppini et al., 1991). Four slices were placed on each membrane. To measure Aβ secretion from cultured transgenic mouse slices, slices were maintained with or without drugs for 4 days before samples were collected for measurements. Individual values represent pooling media from the subsequent 9 days. Aβ was measured using a two-site ELISAs that specifically detect the C terminus of Aβ, as previously described (Tomita et al., 1997). For transient APP overexpression studies, Aβ was measured from media of infected slices 24 hr after infection. For whole-cell electrophysiology, slices were allowed to express recombinant protein for ~24 hr after infection, unless indicated otherwise. Slices were then transferred to a recording chamber and perfused with solution (22°C–25°C) containing 119 mM NaCl, 2.5 mM KCl, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 11 mM glucose, and 0.002 mM 2-chloroadenosine, at pH 7.4 and gassed with 5% CO_2/95% O_2. 100 μM picrotoxin was included in the bath when measuring AMPA or NMDA responses; 20 μM NBQX and 100 μM D,L AP5 were added instead of picrotoxin in experiments measuring inhibitory currents. 2-chloroadenosine was included to prevent bursting. Patch recording pipettes (3–6 MΩ) were filled with intracellular solutions containing 115 mM cesium methanesulfonate, 20 mM CsCl, 10 mM HEPES, 2.5 mM $MgCl_2$, 4 mM Na_2ATP, 0.4 mM Na_3GTP, 10 mM sodium phosphocreatine, and 0.6 mM EGTA, at pH 7.25. Whole-cell recordings were obtained simultaneously from two postsynaptic CA1 neurons (typically one infected expressing GFP), and signals were amplified with Axopatch-1D amplifiers (Axon Instruments). Synaptic responses were evoked by one or two bipolar electrodes with single voltage pulses (200 μs, less than 20V). The stimulating electrodes were placed over Schaffer collateral fibers ~300–500 μm from the recorded CA1 cells. Stimulus level was set to produce a synaptic response of ~40 pA in the control cell. Synaptic responses at −60mV, 0mV, and +40mV were averaged over 50–100 trials. AMPA receptor-mediated response was measured by averaging a 5 ms window about the peak response at −60mV; GABA receptor-mediated response was measured by averaging a 5 ms window about the peak response at 0mV. NMDA receptor-mediated response was measured by averaging a 10 ms window 150 ms after the stimulus artifact of responses recorded at +40mV. For experiments measuring synaptic responses from distant uninfected cell pairs at positions [0,0] and [x,y], the stimulation electrode was placed at a site [x′,y′] equidistant for the two cells and a distance (~300 μm) from the cell body layer using a simple quadratic formula. Coordinates were obtained using a Mitutoyo digital micrometer controlling stage position. Miniature EPSC events were recorded in the presence of 1 μm TTX (no adenosine) and analyzed using the Mini-Analysis software (Synaptosoft). At least 200 events were obtained from each cell. Paired pulse facilitation was elicited by using an interstimulus interval of 50 ms. For whole-cell LTP experiments, potentiation was induced by pairing 2 Hz stimulation with depolarization of the postsynaptic neuron to 0mV for 120 s; recordings were maintained for at least 35 min after pairing. The EPSC amplitude after pairing was normalized to the average amplitude of 20–30 sweeps before pairing. For field potential experiments, hippocampal slices were prepared from P14–P21 animals. Slices were preincubated for 1 hr in drug (1 μM L-685,458) or vehicle (0.1% DMSO) and placed in a recording chamber containing solution indicated above (no 2-chloroadenosine). Responses were evoked by alternating stimuli through two bipolar stimulating electrodes (1V–10V, 200 μs) and recorded with glass electrodes placed in CA1 s. radiatum and amplified with Cyberamp 320 (Axon Instruments). Responses from four slices were recorded simultaneously; drug and no drug experiments were interleaved. All results are reported as mean ± SEM, and statistical significance was set at $p < 0.05$. Statistical differences of

the means were determined using Wilcoxon for paired measurements and t test for nonpaired measurements. To compare the effects on transmission produced by two different APP constructs, a cumulative distribution of response ratios (response in infected cell/response in control cell) were generated for each construct. The Kolmogorov-Smirnov (K-S) test was used to determine statistically significant differences between the two cumulative distributions.

Immunoblot Analysis

Organotypic slices from APP_{SWE} transgenic mice or wild-type rats were solubilized, and extracts were prepared for Western blot analysis (Sisodia et al., 1993). APP fragments were identified by the cytoplasmic terminus antibody CT15, which recognizes full-length APP as well as CTFs, or the β-cleavage site-specific antibody 3D6 (Bacskai et al., 2001). Secreted $APP^{s\beta}$ measurements were carried out by immunoprecipitating $APP^{s\beta}$ from ~500 μ of culture media using antibody 54, which specifically recognizes the C terminus of $APP_{SWE}{}^{s\beta}$ (Howland et al., 1998), and subsequently blotting using antibody 22C11, which recognizes the N terminus of APP. Quantifications of immunoblots were done by enhanced chemiluminescence (ECL; NEN) and densitometric scanning of the films under linear exposure conditions.

Immunohistochemistry/Imaging

Immunohistochemistry was performed on organotypic slices after 1–2 days of infection with the wild-type APP IRES construct that contained a c-tail myc tag. Tissue was fixed in 4% paraformaldehyde solution overnight at 4°C and blocked in PBS containing 10% horse serum, 0.2% sodium azide, and 0.1% triton overnight at 4°C. Slices were incubated in anti-c-Myc (Calbiochem) in blocking buffer overnight at 4°C. Following a 2 hr room-temperature incubation with a biotin-conjugated secondary antibody, avidin-Texas red was added for 2 hr at room temperature. Immunohistochemistry was imaged using a custom built two-photon laser scanning microscope.

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