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Deciphering the Molecular Basis of Memory Failure in Alzheimer's Disease

Review

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Acutely developing lesions of the brain have been highly instructive in elucidating the neural systems underlying memory in humans and animal models. Much less has been learned from chronic neurodegenerative disorders that insidiously impair memory. But the advent of a detailed molecular hypothesis for the development of Alzheimer's disease and the creation of compelling mouse models thereof have begun to change this situation. Experiments in rodents suggest that soluble oligomers of the amyloid β protein (Aβ) may discretely interfere with synaptic mechanisms mediating aspects of learning and memory, including long-term potentiation. In humans, memory impairment correlates strongly with cortical levels of soluble Aβ species, which include oligomers. Local inflammatory changes, neurofibrillary degeneration, and neurotransmitter deficits all contribute to memory impairment, but available evidence suggests that these develop as a consequence of early Aβ accumulation. Accordingly, attempts to slow memory and cognitive loss by decreasing cerebral Aβ levels have entered human trials.

Since before the time of Broca and Wernicke, students of the nervous system have sought to understand normal function by meticulously analyzing dysfunction. The study of disease has provided crucial information about the structure and activities of the healthy brain. In the case of memory, acute brain lesions—usually vascular or traumatic in origin—have yielded powerful insights into some of the networks and mechanisms underlying the formation, consolidation, storage, and retrieval of different forms of human memory. Less has been learned by studying chronic neurodegenerative diseases, in part because they develop very slowly and produce complex mixtures of cognitive symptoms, but primarily because their underlying pathophysiology has remained inaccessible.

But this situation is changing quickly. In the past decade or so, genes causing familial forms of some dementing disorders have been identified, protein pathways involving the gene products have been delineated, and specific treatments directed at these pathways have even begun to enter human trials. Engineered mouse models have enabled a controlled temporal dissection of the pathogenic processes that cannot be achieved by studying human brain tissue at the end of a disease. And increasingly finer anatomical, biochemical, and electrophysiological analyses have focused on early stages of disease evolution—in mice and sometimes even in humans who have died (for other reasons) in the presymptomatic or initial clinical phase of a disorder.

Perhaps the example of greatest relevance to understanding the neurobiology of memory arises from the effort to decipher Alzheimer's disease. In contrast to other syndromes of cognitive failure in humans, such as frontotemporal dementia, multi-infarct dementia, Creutzfeld-Jacob disease, and Lewy body dementia, Alzheimer's disease characteristically produces a remarkably pure impairment of declarative memory in its earliest stages. If one could understand precisely in which neural circuits and by what molecular mechanisms this insidious loss of memory evolves, one might simultaneously derive information about the requirements for normal memory function in adult humans and learn about ways to prevent this catastrophe. Here, we will attempt to distill a rapidly expanding and often bewildering array of experimental and clinical observations into a conceptual model of why memory is impaired in victims of Alzheimer's disease. Then, we will discuss what one might do about it.

The Tragedy of Alzheimer's Disease

Few diagnoses in medicine bring more anguish and foreboding to patient and family than does Alzheimer's disease (AD). This most common of the late-life dementias slowly robs individuals of their most human qualities—memory, insight, judgment, abstraction, and language. And beyond the personal devastation of this ultimately fatal disorder, its commonness produces a societal burden of major proportions. In the year 2000, there were an estimated 4.5 million persons with AD in the United States, and this number is set to triple to 13 million or more by 2050 if no therapy intervenes (Hebert et al., 2004). Based on such estimates in the American population, there may already be well over 30 million victims worldwide. The prevalence of AD rises steadily with age, affecting roughly 1% to 3% of the American population between ages 60 and 70, some 3% to 12% of those between 70 and 80, and upward of 25% to 35% of those over 85 (Evans et al., 1989; Kukull and Bowen, 2002).

The precise onset of clinical AD is very difficult to discern by both patient and family. The earliest symptoms are often manifested as subtle, intermittent deficits in the remembrance of minor events of everyday life, referred to as loss of episodic memory. (The nature of the memory deficits in both AD patients and nondemented aged subjects and their neuroanatomical correlates are reviewed elsewhere in this volume [Buckner, 2004, this issue of *Neuron*].) Early warning signs are frequently

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dismissed as a normal aspect of aging, but as memory loss slowly accelerates, the potential gravity of the situation becomes apparent. Usually, new patients present to the physician in excellent neurological condition, with no deficits other than difficulty encoding some new memories. Speech and language, muscle tone and strength, sensation and reflexes are all initially normal. After many months of gradually progressive impairment of first declarative and then also nondeclarative memory, other cognitive symptoms appear and slowly advance. Over a further period of years or even a decade or more, a profound dementia develops that affects multiple cognitive and behavioral spheres and is often accompanied by extrapyramidal motor signs, slowed gait, and incontinence (Morris and Rubin, 1991; Romanelli et al., 1990). Death usually comes by way of minor respiratory complications, such as aspiration or pneumonia, often in the middle of the night.

Pathologically, the Alzheimer brain at end stage is characterized by atrophy of the hippocampal formation and cerebral cortex and ventricular enlargement, all greater than expected for age. Microscopically, there are decreases in the numbers of neuronal cell bodies in the limbic and association cortices and in certain subcortical nuclei that project to them (e.g., Gomez-Isla et al., 1997; Khachaturian, 1985; Uylings and de Brabander, 2002), although this perikaryal loss can be difficult to appreciate without performing formal stereological quantification. The most obvious and diagnostic microscopic changes in the AD brain are the senile (amyloid) plaques and neurofibrillary tangles (NFT) to which Alzheimer first called attention (Alzheimer, 1907; Kidd, 1964; Terry, 1963). These two lesions usually occur in very substantial numbers in the hippocampus, amygdala association cortices, and certain subcortical nuclei, and they are often accompanied by variable numbers of amyloid-bearing meningeal and cortical microvessels (i.e., congophilic amyloid angiopathy [CAA]). Staining AD brain sections with silver-protein solutions like Bielschowsky's silver impregnation reveals the NFT and the abnormal axons and dendrites (dystrophic neurites) that surround many of the amyloid plaques but that are also scattered widely in the cortical neuropil. By electron microscopy, NFT and some dystrophic neurites can be seen to contain bundles of paired, helically wound ~10 nm filaments (PHF) intermixed with some straight ~10 nm filaments. The highly insoluble filaments of the NFT may be left behind as "ghost tangles" following the death of the neurons in which they originally formed (Braak et al., 1994).

The principal subunit of the PHF is the microtubule-associated protein tau, which undergoes hyperphosphorylation and detachment from microtubles to form these abnormal filaments (for reviews, see Lee et al., 2001; Mandelkow et al., 2003). Inheritance of missense or splicing mutations in the human tau gene causes rare but devastating forms of frontotemporal dementia (Hutton et al., 1998; Lee et al., 2001; Poorkaj et al., 1998; Spillantini et al., 1998), whereas the tau that accumulates in AD is invariably wild-type. The plaque and vascular amyloid deposits of AD are principally composed of the 42 and 40 residue amyloid β proteins (Aβ) that are generated constitutively by sequential proteolysis of the β-amyloid precursor protein (APP) by the β- and γ-secretase enzymes (Glenner and Wong, 1984; Haass et al., 1992; Kang et al., 1987). Antibodies to Aβ have revealed innumerable deposits in the brains of AD patients—and to a lesser extent normal aged humans—that are not fibrillar (i.e., are not amyloid per se) but rather comprise amorphous, granular masses of apparently prefibrillar forms of Aβ (called diffuse plaques) (Dickson, 1997; Duyckaerts et al., 1998).

Alzheimer's Disease as a Disorder of Synaptic Function

Virtually since the time of Alzheimer, neuropathologists have sought to establish semiquantitative correlations between the progressive memory and cognitive symptoms of AD and the morphological alterations found in cortical biopsies and in the autopsied brain. Many studies have examined the relationship between cognitive impairment and plaque and tangle counts, and while in general, the number of NFTs correlates better with severity of dementia than the number of amyloid plaques, arguably the best statistical correlations occur between measures of synaptic density and degree of dementia (e.g., Coleman and Yao, 2003; DeKosky and Scheff, 1990; Terry et al., 1991). Quantification using electron microscopy or immunohistochemical staining for synaptic markers has documented significant decreases in synaptic density in the association cortices and hippocampus of AD brain (Bertoni-Freddari et al., 1989; Davies et al., 1987; DeKosky and Scheff, 1990; Masliah et al., 1990, 2001; Sze et al., 1997; Terry et al., 1991). Moreover, the decrease in synapse number and density seems disproportionate to the loss of neuronal cell bodies (Davies et al., 1987; DeKosky and Scheff, 1990; Bertoni-Freddari et al., 1996), suggesting that pruning of synaptic endings may precede the demise of the neuron in the disease process. Furthermore, some changes in the brains of AD patients and APP transgenic mice suggest that synaptic function is compromised prior to the physical degeneration of the synapses (e.g., Palop et al., 2003; Westphalen et al., 2003; Yao et al., 2003).

Amyloid β Protein as an Instrument of Synaptic Attack

In the AD brain after death, one can observe many abnormalities that would be expected to have interfered with memory function. These include swollen and tortuous dendrites and axons, activated microglia and reactive astrocytes containing inflammatory mediators (e.g., cytokines, acute phase proteins) and free radicals, neurofibrillary tangles, and deficits of several neurotransmitters attributable to synaptic and perikaryal loss. How can one begin to make sense of the number and complexity of potentially adverse influences on memory function? And how might one decipher the cause of the earliest memory symptoms, not only in AD but also in the more subtle amnestic syndrome of mild cognitive impairment (MCI), a frequent prelude to—or first clinical stage of—AD? For many investigators, answers to these two questions have emerged from detailed analyses of the molecular pathology of these disorders coupled with identification of genetic factors that predispose strongly to the development of AD. And while controversy re-

mains, one can synthesize the results of such studies into a hypothesis that the gradual accumulation of Aβ in brain regions important for memory initiates the AD syndrome.

Diverse lines of evidence now suggest that Aβ plays a central role in the pathogenesis of neuronal dysfunction in AD (for reviews, see Hardy and Allsop, 1991; Hardy and Selkoe, 2002; Selkoe, 1991). The salient points of support for the "amyloid hypothesis" (more correctly, the Aβ hypothesis) of AD can be summarized briefly. First, Aβ is the subunit of the amyloid that is progressively deposited in myriad neuritic plaques in the limbic and association cortices of all AD patients (Glenner and Wong, 1984; Masters et al., 1985). Second, synthetic Aβ peptides are toxic to hippocampal and cortical neurons, both in culture and in vivo (e.g., Geula et al., 1998; Lorenzo and Yankner, 1994; Pike et al., 1991). Third, the *APP* gene is on human chromosome 21q (Goldgaber et al., 1987; Kang et al., 1987; Robakis et al., 1987; Tanzi et al., 1987), and its duplication leads to the typical AD neuropathology that invariably develops in middle-aged patients with trisomy 21 (Down's syndrome) (Mann et al., 1984; Mann, 1988). Importantly, in a rare case of the translocation form of Down's syndrome in which the distal location of the chromosome 21q breakpoint left the patient diploid for the *APP* gene, no signs of dementia developed, and amyloid deposition and AD-type neuropathology were essentially absent from the brain upon death at age 78 (Prasher et al., 1998). Fourth, inherited mutations in the *APP* gene that all localize within or immediately flanking the Aβ region alter the amounts or aggregation properties of Aβ and are sufficient to precipitate premature AD (Goate et al., 1991; Levy et al., 1990). Fifth, inherited mutations within the presenilin (*PS*) 1 and 2 genes increase the Aβ42/Aβ40 ratio throughout life and cause very early and aggressive forms of AD (Lemere et al., 1996; Levy-Lahad et al., 1995; Rogaev et al., 1995; Scheuner et al., 1996; Sherrington et al., 1995). In this regard, presenilin has been found to be the active site component of the protease (γ-secretase) which generates Aβ (Esler et al., 2000; Li et al., 2000; Wolfe et al., 1999). Sixth, inheritance of one or two ϵ4 alleles of apolipoprotein E (*Apo E*) is a strong genetic risk factor for AD (Corder et al., 1993; Saunders et al., 1993; Strittmatter et al., 1993) and increases cerebral Aβ burden in humans (Rebeck et al., 1993; Schmechel et al., 1993). And seventh, mice transgenic for mutant human APP show a time-dependent increase in extracellular Aβ and develop certain neuropathological and even behavioral changes reminiscent of AD (Games et al., 1995; Hsia et al., 1999; Hsiao et al., 1996; Johnson-Wood et al., 1997; Moechars et al., 1999; Sturchler-Pierrat et al., 1997).

Both the APP mutations that flank the C terminus of the Aβ region and the mutations in PS1 and PS2 selectively increase the cellular production of Aβ terminating at amino acid 42 (Borchelt et al., 1996; Duff et al., 1996; Scheuner et al., 1996; Citron et al., 1997). The increase in Aβ42 is particularly noteworthy, because this form of Aβ is far more prone to oligomerization and fibril formation than is the more abundantly produced Aβ40 peptide (Bitan et al., 2003; Burdick et al., 1992; Jarrett et al., 1993). Five APP point mutations occur within the Aβ sequence (Cras et al., 1998; Kamino et al., 1992; Maat-Schieman et al., 1994; Maat-Schieman et al., 1992; Tagliavini et al., 1999), and in each case, these increase the steady-state levels of Aβ and/or its propensity to polymerize (Clements et al., 1993; De Jonghe et al., 1998; Fraser et al., 1992; Van Nostrand et al., 2001; Watson et al., 1999; Nilsberth et al., 2001). Studies of genetically manipulated mice reveal that the apoE protein, particularly the E4 isoform, facilitates the formation or stability of Aβ fibrils (Fagan et al., 2002), and apoE may play an important role in the extracellular clearance of Aβ (DeMattos et al., 2004; Koistinaho et al., 2004). In short, all four confirmed genetic factors underlying inherited forms of AD increase the production and/or accelerate the aggregation of Aβ, and this can be detected in humans well before the onset of clinical symptoms.

Despite the evidence summarized above, the Aβ hypothesis remains controversial (Lee et al., 2003; Mesulam, 1999; Neve and Robakis, 1998), not least because the quantity and temporal progression of amyloid plaques do not show a simple relationship to the clinical progression of the disease (Braak and Braak, 1998). Extensive cortical plaques, mostly of the diffuse type, are detected in a significant proportion of the nondemented elderly (Knopman et al., 2003). We will now review a range of studies that suggest that the relatively weak correlation between cerebral amyloid plaque burden and severity of memory and cognitive impairment may be explained by evidence that Aβ neurotoxicity can be mediated by multiple different assembly forms of the peptide and that impaired memory may be attributable, at least in part, to soluble oligomers that can initiate downstream changes. This concept is worthy of focus in view of very recent information that therapeutically lowering cortical Aβ levels in some AD patients may actually be associated with stabilization of memory and cognitive decline (Hock et al., 2003; Nicoll et al., 2003; Gilman et al., 2004).

Studies of Human Brain Tissue and AD Mouse Models Suggest that Soluble Forms of Aβ Perturb Synaptic Form and Function

An early hint that soluble, nonfibrillar assemblies of Aβ might play a role in memory impairment came from analyses of human brains that demonstrated robust correlations between cortical levels of soluble Aβ and the extent of synaptic loss and severity of cognitive impairment (Lue et al., 1999; McLean et al., 1999; Wang et al., 1999). In such studies, the term soluble Aβ is an operational definition, embracing all forms of Aβ that remain in aqueous solution following high-speed centrifugation of brain extracts (Kuo et al., 1996; Lue et al., 1999; McLean et al., 1999; Wang et al., 1999). To date, most analyses of soluble Aβ levels have employed quantification by ELISAs that cannot disclose the aggregation state of the species detected. Thus, while such studies cannot attribute synaptic and cognitive changes to a specific assembly form of Aβ, the solubility of the Aβ species in aqueous buffer following ultracentrifugation (typically, $\geq$100,000 $\times$ g for $\geq$1 hr) indicates that the samples are free of amyloid fibrils, which pellet quantitatively at these forces. In one study, when this buffer-soluble fraction of AD cerebral cortex was examined by sensitive Western blotting, not only monomeric Aβ (4 kDa) but also SDS-

stable oligomers (~8 and ~12 kDa) were detected (McLean et al., 1999). Similar oligomers have also been detected in the hippocampal CA1 region and entorhinal cortex of humans in the absence of amyloid plaques and, in many cases, in the absence of NFTs as well (Funato et al., 1999). The latter result suggests that the accumulation of Aβ oligomers may occur very early in the disease process in humans.

Given the many obstacles to assessing disease progression dynamically in the human brain, significant effort has been expended to create mouse models that might replicate aspects of AD pathogenesis. A sizeable number of mouse lines transgenic for human APP have been generated. Most of these models recapitulate some, but not all, of the neuropathological features of the human disease. Experience suggests that mouse models with expression of human APP well above endogenous levels usually show pathological and behavioral abnormalities, but the amount of APP overexpression required to induce easily detectable phenotypes depends upon the background strain of the mouse (Hsiao, 1998). Typically, animals with APP levels four to five times higher than endogenous develop amyloid plaque pathology similar to that observed in AD, with the Aβ42/40 peptide ratio apparently being the critical determinant (Mucke et al., 2000). Both diffuse and mature (fibrillar) plaques are usually present in such mice, with an age-dependent increase in the number and density of such plaques being an invariant feature (Games et al., 1995; Hsia et al., 1999; Hsiao et al., 1996; Johnson-Wood et al., 1997; Moechars et al., 1999; Sturchler-Pierrat et al., 1997). Mature plaques are stained by Thioflavin S and silver solutions and display birefringence under polarized light upon labeling with Congo red. These mouse plaques, like those in AD, also show reactive astrocytes (stained by antibodies to glial fibrillary acidic protein), activated microglia, and surrounding neuritic dystrophy, but no PHF in neurites or tangles have been observed without also overexpressing mutant human tau (Games et al., 1995; Hsia et al., 1999; Hsiao et al., 1996; Moechars et al., 1999; Sturchler-Pierrat et al., 1997). "Bigenic" mice expressing mutant human APP plus human tau bearing the P301L mutation that causes a form of frontotemporal dementia (Lewis et al., 2001) or "trigenic" mice expressing mutant APP, mutant PS1, and P301L tau (Oddo et al., 2003) develop NFTs reminiscent of those seen in AD and at a rate much accelerated compared to mice expressing P301L tau alone. The rapidity of evolution and spatial pattern of Aβ deposition varies among different APP transgenic lines, being generally dependent on the transgene promoter, the level of expression achieved, and the mouse strain.

In most APP transgenic models, neuronal loss has not been observed by the use of conventional stereological techniques (e.g., Irizarry et al., 1997a, 1997b). However, at least one mouse line (called APP23) that expresses mutant human APP exhibits significant neuronal loss in the CA1 region of hippocampus at age 14–18 months (Calhoun et al., 1998). In these mice, Aβ is deposited almost exclusively in the form of Thioflavin-positive plaques (Sturchler-Pierrat et al., 1997), and the cell loss is observed primarily in the vicinity of such hippocampal deposits. Additional neuronal loss that did not reach statistical significance was seen in the neocortex, where the Aβ plaques disrupted local cytoarchitecture, and in certain subregions such as the piriform and entorhinal cortices (Calhoun et al., 1998). Similar selective neuronal loss in CA1 of hippocampus and certain subregions of the neocortex has been observed in AD brains (Calhoun et al., 1998), suggesting that the predominance of mature (amyloid fibril-rich) plaques in the APP23 line leads to some neuronal loss in areas of vulnerability. In other APP transgenic lines, more diffuse and less mature plaques occur than in the APP23 mice, and such lines generally do not show measurable loss of neuronal cell bodies (Calhoun et al., 1998; Games et al., 1995; Irizarry et al., 1997b).

In accord with these observations, a study using triple immunolabeling confocal microscopy and cross-correlation density map analysis reported a significant reduction in neuronal density (as assessed by staining for Neuro N) around Thioflavin S-positive Aβ deposits in AD brain and also in the brains of 12-month-old "bigenic" mice that express mutant human PS1 plus APP (Urbanc et al., 2002). In these mice, significant decreases in neuronal density were detected within plaques, in proportion to the density and size of the deposits. Modeling the spatial relationship between neurons and Thioflavin S-positive deposits suggested that Aβ fibrils were associated with loss of neurons within the plaque, but this toxicity did not extend beyond the deposit itself. Because Thioflavin S-positive plaques occupy only ~2% of the mouse cortical area, the resultant neuronal loss is modest and would not be easily detected with standard stereological methods. Similarly, in AD brains, Thioflavin S-positive amyloid plaques have been estimated to occupy only around 4% of cortical area (Urbanc et al., 2002); as a result, the local neurotoxicity of these plaques alone cannot account for the total neuronal loss observed in AD brains. Although it is likely that fibrillar Aβ in mature plaques confers cytotoxicity, it is worth emphasizing that amyloid fibrils are dynamic structures and may actually act as local reservoirs of potentially cytotoxic low molecular weight Aβ assemblies. It remains unclear which additional Aβ assemblies and/or other molecular events may mediate the substantial neuronal loss observed in late-stage AD brains (Everall et al., 1997; Gomez-Isla et al., 1997) or mediate the deficits in synaptic markers observed in certain APP transgenic mice prior to the appearance of Aβ deposits.

In a mouse line transgenic for APP bearing the Val717-Phe FAD mutation (designated "lond 2 mice"), it took ~12 months for Aβ plaques to develop, and yet from 3 months onward, the animals showed cognitive impairment and decreased long-term potentiation (LTP), an electrophysiological correlate of aspects of learning and memory (Moechars et al., 1999). When these lond 2 mice were crossed with neuron-specific PS1-deficient mice, the offspring showed no Aβ deposition at age 18 months, and LTP was virtually normal (Dewachter et al., 2002). The latter finding suggests a critical role for Aβ per se in the block of LTP, because eliminating PS1 (and thus markedly lowering γ-secretase activity) still leaves production of full-length APP and its major soluble derivative (APPs-α) unchanged and actually elevates levels of the C99 and C83 C-terminal fragments of APP. These results help address the appropriate concern that APP

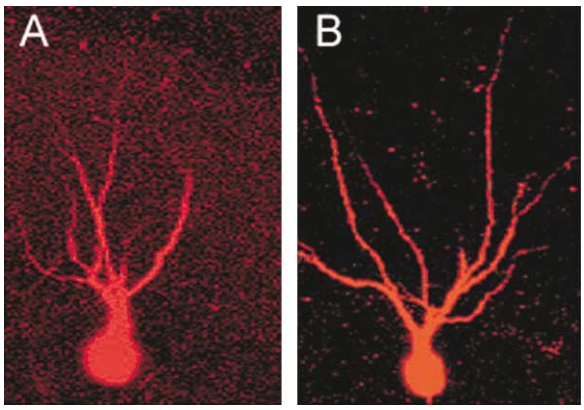

Figure 1. Superficial Granule Cells of the Dentate Gyrus Show Reduction in Dendrite Length Many Months Before Aβ Deposits Are Detected

Superficial granule cells from PD-APP mice (A) exhibit a significant reduction in extent of dendritic trees in comparison to nontransgenic control mice (B). (Adapted from Wu et al., 2004).

transgenic mice overproduce not only Aβ but also APP and APPs-α, making it difficult to attribute deficits to Aβ alone. However, the enhancement of neuropathological and behavioral phenotypes in APP transgenic mice achieved by coexpressing Aβ42-elevating PS1 missense mutations (which do not alter APP and APPs-α levels) again implicates Aβ in such deficits.

In support of the occurrence of deficits in synaptic function that precede the development of Aβ deposits, young (4- to 5-month-old) PD-APP transgenic mice (which express V717F mutant human APP) were found to have enhanced paired-pulse facilitation, distorted responses to high-frequency stimulation bursts, and impaired LTP (Larson et al., 1999; N. Shinsky et al., 2002, Soc. Neurosci., abstract Volume *28*). Moreover, morphological analyses of 3-month-old PD-APP mice revealed a structural compromise of dentate granule cells many months in advance of amyloid deposition (Figure 1). There was a ~12% decrease in total dendrite length of granule cells, with superficial granule cells in the posterior region of the dorsal blade showing a massive 32% reduction in dendrite length (Wu et al., 2004).

Further support for a role of soluble Aβ species in Alzheimer-like neuronal dysfunction comes from the occurrence of progressive learning deficits, along with declines in synaptic transmission and synaptophysin and MAP-2 immunostaining, in the hippocampal CA1 region of APP V717F transgenic mice at times well before any amyloid plaques are observed (Hsia et al., 1999; Mucke et al., 2000; Palop et al., 2003). These deficits then increased with age but did not correlate with plaque number. One study reported a change in basal synaptic transmission without a change in LTP (Hsia et al., 1999), whereas another in a separate mouse line bearing the same APP mutation found no change in basal synaptic transmission but an impairment of LTP (Chapman et al., 1999). In the latter mice, no perikaryal loss was observed (Irizarry et al., 1997b), and the changes in LTP were ascribed to functional changes in synapses, whereas in the former mice, there was evidence of synaptic loss without a change in the electrophysiological properties of the remaining synapses. Examining APP transgenic as well as APP + PS "bigenic" mouse lines, Dickey and colleagues reported that certain gene products associated with synaptic plasticity and memory consolidation were suppressed at a time when synaptophysin expression was still normal (Dickey et al., 2003, 2004). These variations among studies could result from discrepancies in experimental design, particularly, differences in the ages at which the mice were tested and from strain differences, as it is known that susceptibility to neuronal injury can vary widely across mouse strains (Schauwecker and Steward, 1997).

It will now be important to compare quantitative immunohistochemistry for synaptophysin and other synaptic proteins to electrophysiological measures of synaptic strength prior to the development of amyloid pathology in various APP transgenic models, to determine to what extent functional changes precede structural changes in synapses. Available data suggest that diffusible, prefibrillar Aβ assemblies may affect both the form and function of synapses in transgenic models, but the effects seem to vary with age, strain, and brain region.

Particularly compelling evidence for neuronal/synaptic functional compromise by Aβ species other than fibrillar plaques arose from a report that deficits of memory function in APP transgenic mice were reversed by a single intraperitoneal injection of anti-Aβ antibodies (Dodart et al., 2002). In these acute (<24 hr) experiments, brain amyloid burden was not decreased (as expected), suggesting that the antibody must be acting on soluble, diffusible species of Aβ and that sequestration or clearing of these intermediates allowed an overnight return to near-normal performance in an object recognition task (Figure 2). Another transgenic model, *C. elegans* that express human Aβ1-42 in body wall muscle using a myosin promoter-Aβ minigene, provides additional evidence that soluble Aβ assemblies are cytotoxic in vivo, as these animals display a paralysis phenotype that develops well before any amyloid deposits are detected (Drake et al., 2003).

Cell-Derived Oligomers of Human Aβ Disrupt Both Synaptic Plasticity and the Memory of Learned Behavior

Although the studies reviewed above suggest that soluble, prefibrillar assemblies can induce early neuronal alterations, the specific nature of the Aβ species mediating these changes and their mechanisms of action have not been defined. To model Aβ-mediated neurotoxicity, many investigators have used synthetic peptides (for a review, see Walsh et al., 2003). At ambient or body temperature and at concentrations ≥10–20 μM, synthetic Aβ1-40 and Aβ1-42 each self-associate to form low-n oligomers, protofibrils, and fibrils. An important caveat when considering the cellular effects of different Aβ assemblies is the highly dynamic nature of Aβ aggregation. Because intermediates can further associate into higher ordered aggregates and fibrils can dissociate, it is difficult to unambiguously ascribe cytopathological activity to a discrete species. Nonetheless, several groups have attempted to isolate prefibrillar synthetic Aβ assemblies and probe their synaptotoxic activity. In 1998, Lambert and colleagues presented the first experi-

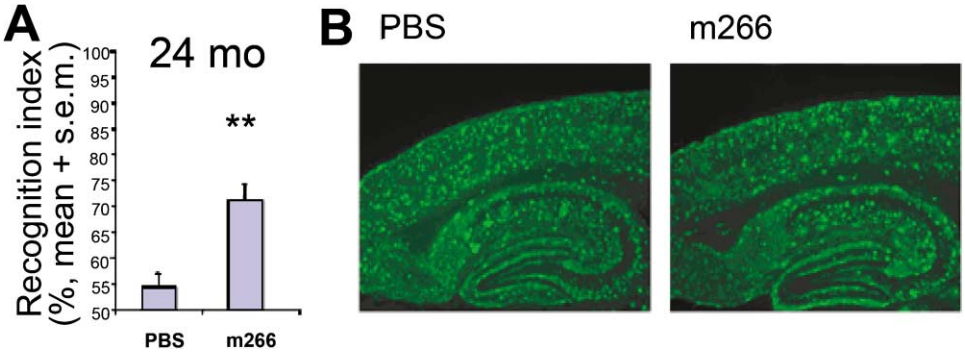

Figure 2. Soluble Prefibrillar Assemblies of Aβ Disrupt Object Recognition Memory

APP transgenic mice (24-month-old) (n = 8 per group) were injected with anti-Aβ monoclonal antibody m266 or PBS once per week for 6 weeks. Behavioral testing was done 3 days after the last injection. Similar results were obtained after just a single injection of the same antibody. (A) Performance in the object recognition task is expressed as a recognition index corresponding to the percentage of time spent exploring a novel object versus a familiar object during the test session. A recognition index of 50% indicates that the mice do not discriminate between the novel and the familiar object. Object recognition memory performance of mice treated with m266 is significantly better ($p < 0.01$, ANOVA) than that of mice treated with PBS. (B) Anti-Aβ immunostained sagittal brain sections from mice treated with PBS (left) or the m266 antibody (right) show that passive immunization did not detectably alter the cerebral burden of Aβ deposits. (Adapted from Dodart et al., 2002).

mental evidence that certain soluble, nonfibril assemblies of synthetic Aβ (which they called Aβ-derived diffusible ligands, or ADDLs) could be neurotoxic (Lambert et al., 1998). ADDLs are formed by incubating synthetic Aβ1-42 in ice-cold Ham's F12 medium, yielding spherical structures of ~5 nm diameter, with the Aβ species migrating on SDS-PAGE at ~4, 8, 16, and 18 kDa. ADDLs have been shown to cause neuronal death in culture, to block LTP (Lambert et al., 1998; Wang et al., 2002), and to inhibit reduction of MTT by neural cell lines (Lambert et al., 1998; Stine et al., 2003). When incubated with organotypic mouse brain slices at 500 nM for 45–60 min, cell loss was not evident, but a near-complete block of LTP was observed (Lambert et al., 1998; Wang et al., 2002). It is conceivable that, during their incubation with neurons, ADDLs may form larger Aβ assemblies; however, the electrophysiological experiments were performed over a short time course (1–2 hr) and at concentrations (~500 nM) well below the critical concentration for synthetic Aβ fibril formation in vitro, suggesting that ADDLs are themselves synaptotoxic.

Assembly intermediates of synthetic Aβ termed protofibrils (PF) can also rapidly alter neuronal function. When viewed by electron microscopy or atomic force microscopy, PF range from spherical assemblies of ~5 nm diameter to short, flexible rods of up to 200 nm in length (Harper et al., 1997; Walsh et al., 1997). Unlike ADDLs, PF can be generated in vitro under a variety of biochemical conditions, and their rate of formation is dependent on Aβ concentration, pH, and ionic strength (Harper et al., 1999). PF appear to behave as true fibril intermediates in that they can both form fibrils and dissociate to lower molecular weight species (Harper et al., 1999; Walsh et al., 1999). Using whole-cell patch-clamp recordings, PF composed of Aβ1-40 induced an instantaneous increase in excitatory postsynaptic currents (EPSCs) in rat cortical neurons (Hartley et al., 1999). Fibril preparations also enhanced EPSCs, whereas monomeric Aβ had no effect. In a whole-cell current-clamp recording mode, application of PF induced an instantaneous increase in action potentials and large membrane depolarizations, indicating that PF can alter membrane excitability (Hartley et al., 1999). This excitability was entirely reversible and was concentration dependent, with activity starting at low micromolar concentrations. Moreover, PF appear to have inherent electrophysiological activities distinct from fibrils, because the addition of the specific NMDA antagonist, D-APV, attenuated PF-stimulated neuronal activity by 72%, whereas the same dose reduced fibril-induced activity by only 38% (Ye et al., 2004). In contrast, the application of the non-NMDA antagonist NBQX produced only a 23% decline in PF-induced activity but decreased fibril-induced activity by some 50%. These data suggest that glutamate receptor channels are involved in PF-induced neuronal excitability and that synthetic PF and fibrils may act, at least in part, via different neurobiological mechanisms.

While the studies just described provide strong evidence that soluble prefibrillar assemblies of synthetic Aβ such as ADDLs and PF can alter synaptic function, there is as yet no confirmation that these species actually occur in nature. As an alternative experimental approach to dissect the biological properties of early Aβ assemblies, we chose to study the activity of naturally produced, cell-derived Aβ oligomers. We took advantage of a cell line expressing mutant (V717F) human APP that generates SDS-stable low-n oligomers intracellularly and secretes a portion of them into the medium. Small aliquots (1.5–5.0 μl) of conditioned medium (CM) containing low- or subnanomolar concentrations of these entirely soluble oligomers were microinjected intracerebroventricularly into rats and found to inhibit the maintenance of hippocampal LTP in vivo (Walsh et al., 2002). Evidence that the failure to sustain LTP was mediated by the Aβ oligomers in the CM emerged from biochemical manipulation of the sample. Immunodepletion of the CM with Aβ-specific antibodies prevented the block of LTP, whereas immunodepletion of the abundant soluble APPs-α derivative had no effect. Most importantly, preincubation of the CM with insulin degrading enzyme (IDE), a protease that efficiently degrades Aβ monomer but not oligomers, did not alter the LTP effect.

Although these results provide direct evidence that diffusible, low-n oligomers of human Aβ, in the complete absence of Aβ monomers, protofibrils, or fibrils, confer "synaptotoxicity," we sought to provide further support for this hypothesis. To this end, we employed size exclusion chromatography (SEC) to fractionate the CM (using nondenaturing, nondisaggregating buffers) and showed that the block of LTP was specifically mediated by the low-n oligomers, not by Aβ monomers or any larger aggregates (Walsh et al., 2004). Taken together, these results demonstrate that a biochemically defined, oligomeric assembly of naturally secreted human Aβ alters hippocampal synaptic plasticity in vivo. The same secreted oligomeric forms of Aβ also inhibit LTP in vitro in hippocampal slices from both rat (Wang et al., 2004) and mouse (Walsh et al., 2004).

Whether LTP is a valid electrophysiological surrogate of learning and memory processes is still contentious (reviewed in Dudai, 2002). Therefore, we proceeded to assess whether an impairment of short-term memory similar to that associated with progressive Aβ accumulation in MCI and early AD could actually be induced directly by soluble Aβ oligomers. To determine the effects of physiological levels of naturally secreted human Aβ upon a complex learned behavior, we again microinjected the CM of the APP-expressing cells into the ventricles of rats. A barrier to studying this problem has been the lack of a sufficiently sensitive assessment procedure capable of measuring transient cognitive changes in rodents over time and treatment conditions. To overcome this problem, we utilized the alternating lever cyclic ratio (ALCR) test, a procedure proven to be 1–2 orders of magnitude more sensitive than previously published methods for measuring drug effects on cognitive function in rats (O'Hare et al., 1996; Richardson et al., 2002). In the ALCR paradigm, rats learn a complex sequence of lever-pressing requirements. The animals must alternate between two levers, switching to the second lever after pressing the first lever enough times to get a food pellet. The number of presses required for each food reward proceeds from 2 to 56, incorporating intermediate values based on the quadratic function, $x^2 - x$. One cycle is an entire ascending and descending sequence of these response requirements (e.g., 2, 6, 12, 20, 30, 42, 56, 56, 42, 30, 20, 12, 6, and 2 presses per food reward). Six such full cycles are presented during each session. Errors are scored when the rat perseveres on a lever after reward, i.e., does not alternate (a "perseveration error"), or when an animal switches levers before completing the required number of presses on that lever (a "switching error"). Rats microinjected with the Aβ-containing CM showed a marked increase in both switching and perseveration errors when tested 2 hr after injection, but recovered to baseline when retested 24 hr later (Cleary et al., 2004). Evidence that this transient interruption of a learned behavior was attributable to Aβ oligomers came from the findings that immunodepleting the CM of Aβ rendered the CM inactive, and, more specifically, that SEC fractions containing oligomers induced the deficits, whereas monomer-containing fractions from the same SEC run had no effect (Cleary et al., 2004). Independently, Kawarabayashi and colleagues reported that the appearance of dimeric Aβ in cortical lipid raft fractions coincides with the first indicators of behavioral compromise in APP transgenic mice (Kawarabayashi et al., 2004), consistent with our finding that dimers and trimers of Aβ can interfere with the memory of a learned behavior.

In view of the mounting evidence that Aβ oligomers can alter synaptic function in vivo, much work is now needed to identify the molecular mediators of these adverse effects. This quest has begun, and certain neuronal cell surface receptors as well as certain second messenger signaling pathways have begun to be implicated (see, for example, Wang et al., 2004; Dickey et al., 2003; Vitolo et al., 2002). It remains to be clarified whether the rather hydrophobic Aβ oligomers operate through one (or a small number of) biochemically specific neuronal receptor(s) or, perhaps more likely, perturb nonspecifically several receptors/channels normally required for triggering signaling events that result in encoding of memories.

How Can We Neutralize the Effects of Soluble Aβ Oligomers on Hippocampal Synaptic Function?

Aβ is produced at discrete sites within living cells as a monomer (Walsh et al., 2000), and it appears to enter rapidly into an equilibrium with dimers and trimers intracellularly (Walsh et al., 2002), a process similar to that described for synthetic Aβ peptides in vitro (Bitan et al., 2003). At least some of these natural, low-n oligomers are highly stable (via strong hydrophobic interactions and/or covalent cross-links), and a portion of these is subsequently secreted from the cell (Luo et al., 2002; Walsh et al., 2002). Importantly, such SDS-stable oligomers have been detected inside cultured fetal human neurons and in human CSF, indicating that they can arise in and be released by human neurons (Walsh et al., 2000). As discussed above, the secreted oligomers have been shown to interact with neurons, altering their normal physiology (Walsh et al., 2002) and even inducing transient impairment of memory (Cleary et al., 2004).

Based on these findings and the other experimental observations reviewed herein, we propose that analogous synaptic changes may contribute to the development of the earliest symptoms of MCI and AD by subtly altering neurotransmission and perhaps initiating synaptic remodeling, akin to the early (preplaque) changes observed in Aβ-overproducing transgenic mice (Hsia et al., 1999; Lanz et al., 2003; Mucke et al., 2000). These effects of diffusible Aβ oligomers could account for the subtle impairments of memory function documented in APP transgenic mice (e.g., Chen et al., 2000; Janus et al., 2000; Morgan et al., 2000) and perhaps in MCI and AD subjects themselves. Thereafter, steadily rising concentrations of soluble monomers and oligomers may allow increasing self-association, leading gradually to first diffuse and then fibrillar extracellular plaques, which could themselves act as reservoirs for diffusible oligomers that may further disrupt neuronal circuits.

In view of these considerations, a particularly attractive therapeutic approach would be to prevent the formation of potentially synaptotoxic oligomers. γ-Secretase inhibitors can markedly decrease Aβ oligomer formation by cultured cells at doses that still allow appreciable monomer production (Walsh et al., 2002), and

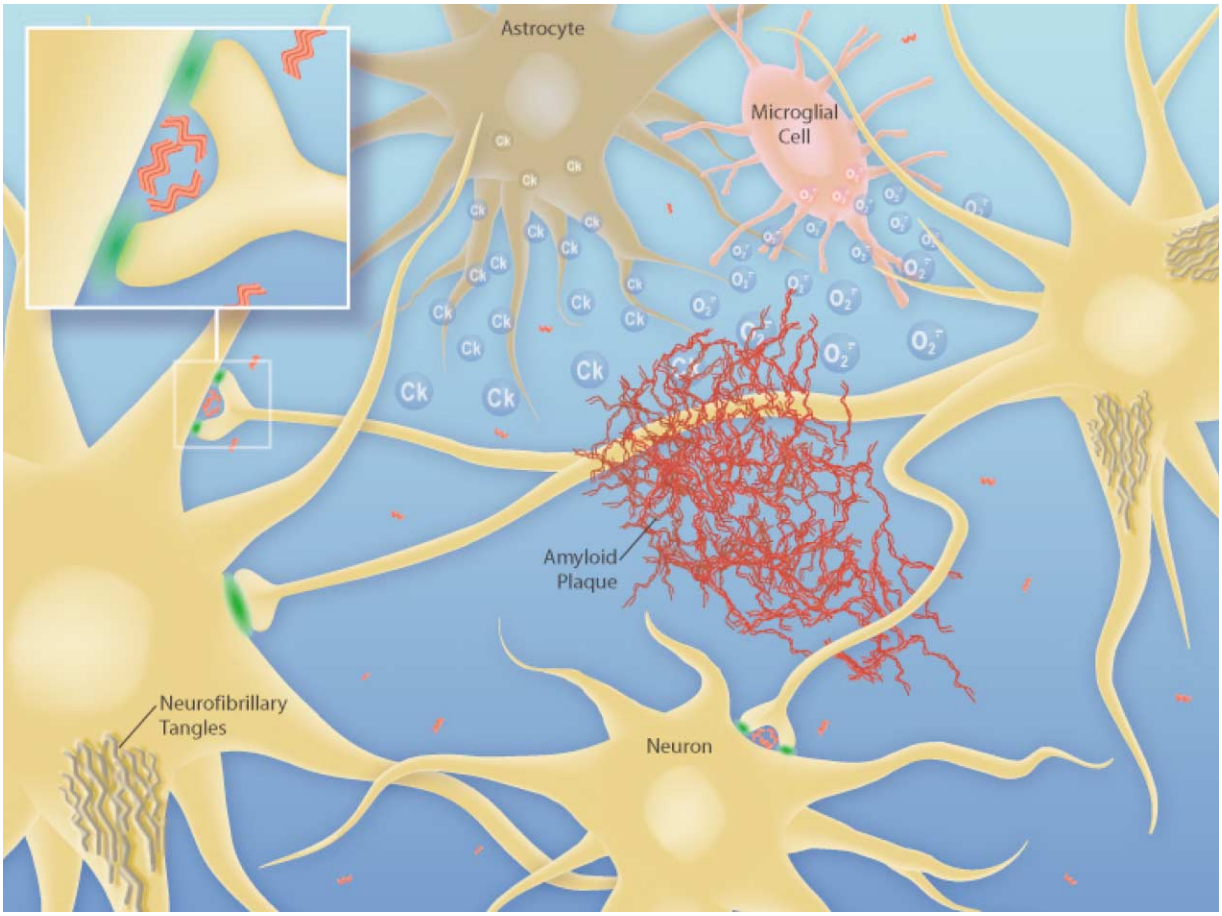

Figure 3. Several Different Pathogenic Events May Contribute to Synaptic Dysfunction in Alzheimer's Disease

Different Aβ assembly forms may mediate diverse cytotoxic effects, including decreased synaptic efficacy, distortion of axonal pathways, shrinkage of dendritic arbors, activation of microglia, free radical release, and inflammatory changes. The cartoon depicts the distortion of axonal trajectories observed within amyloid plaques and the periplaque activation of astrocytes, resulting in the release of various cytokines (Ck), and microglia, resulting in the generation of superoxide radicals ($O2^{-}$). Disruption of synaptic efficacy by diffusible, low-n oligomers of Aβ is depicted as a decrease in normal transmission at synapses (green cloud) due to the presence of Aβ dimers and trimers in the cleft that can contact synaptic plasma membranes. All Aβ species are shown in red, with amyloid plaques shown as an interwoven mass of fibrils and soluble Aβ dimers and trimers depicted as stacked W-shaped structures (suggesting their β sheet-rich structure).

cell-penetrant inhibitors of β-secretase (e.g., Chang et al., 2004) or other agents that reduce intracellular and/or extracellular monomer levels below the critical concentration needed for oligomerization could have similar effects. Thus, exploitation of the preclinical approaches to Aβ oligomerization we review above could help identify the minimal oligomer-preventing concentrations of different amyloid-lowering compounds and thus decrease the likelihood of adverse effects in humans. Although no physiological function has been confirmed for the Aβ monomer itself, substantial or complete depletion of monomers in vivo could potentially result in adverse effects. In contrast, Aβ oligomers presumably arise solely as a pathological event upon elevation of monomer concentrations in advanced age and during the prodromal phase of MCI and AD. Another antiamyloid approach for treating AD is the use of peptidomimetics or other small molecules to inhibit Aβ aggregation. For example, certain hydroxyanaline derivatives are capable of inhibiting intracellular oligomer formation and blocking the oligomer-mediated inhibition of hippocampal LTP (Walsh et al., 2004).

The most clinically tested amyloid-directed therapy, Aβ immunization (Hock et al., 2003; Nicoll et al., 2003), has been shown in multiple studies of APP transgenic mice to reduce cerebral Aβ levels and plaque burden and thus decrease amyloid-associated gliosis and neuritic dystrophy and alleviate memory impairment (e.g., Bard et al., 2000; Chen et al., 2000; Morgan et al., 2000; Schenk et al., 1999; Weiner et al., 2000). In recent unpublished experiments, we found that the ventricular coinjection of anti-Aβ monoclonal antibodies with soluble Aβ oligomers in vivo can rescue the oligomer-mediated block of LTP and also that rats with high circulating levels of endogenous anti-Aβ antibodies (following immunization) are similarly protected (I. Klyubin, D.M.W., D.J.S., and M. Rowan, unpublished data). These results suggest that anti-Aβ antibodies could bind and help clear soluble oligomers of Aβ, so that the latter are no longer present at sufficient concentrations to alter syn-

aptic physiology. Such a mechanism could explain the rapid reversal of cognitive deficits in APP transgenic mice treated acutely with an Aβ monoclonal antibody (Dodart et al., 2002).

Many of the experiments reviewed herein address the effects of the extracellular application of Aβ oligomers, but one cannot exclude the possibility that the intracellular oligomers detectable in neurons (Morishima-Kawashima and Ihara, 1998; Skovronsky et al., 1998; Takahashi et al., 2004; Walsh et al., 2000) also impact upon synaptic function. However, the fact that antibody-mediated clearance of Aβ from the brain was effective in blocking age-dependent memory deficits in transgenic mice (e.g., Janus et al., 2000; Morgan et al., 2000) suggests that intraneuronal Aβ oligomers (which should not be readily accessed by the antibodies) may contribute less to synaptic dysfunction than their secreted counterparts. In any event, the use of Aβ-lowering compounds (e.g., β- or γ-secretase inhibitors), which decrease both intra- and extracellular oligomer levels while still allowing significant monomer production, appears particularly desirable.

Conclusion

Although there is now substantial evidence that subtle, intermittent impairment of memory can be mediated by the synaptic effects of diffusible Aβ oligomers, larger Aβ assemblies—including protofibrils and fibrils—are also likely to contribute to neurotoxicity in vivo. Indeed, in the human brain, it is likely that multiple Aβ assemblies that are in dynamic equilibrium simultaneously alter neuronal, astrocytic, and microglial function and that different toxic effects may occur virtually concurrently in various regions of the cerebral cortex and certain nuclei projecting to it (Figure 3). So why focus on Aβ oligomers? The answer is two-fold: low-n oligomers are the earliest pathogenic assemblies of Aβ formed, and their functional effects appear to be reversible. Hence, any therapeutic approach that prevents the formation of Aβ dimers should potentially allow the recovery of synaptic functions, such as LTP, that have been shown to be altered by oligomers. At the same time, such therapies should prevent the formation of larger aggregates, thus ameliorating the apparent downstream effects of various toxic Aβ assemblies, such as microgliosis, astrocytosis, NFT formation, and neurotransmitter deficits. Only the evaluation of such approaches in humans can determine whether the mechanisms discussed here contribute to the insidious decline of memory in aged humans with mild cognitive impairment and Alzheimer's disease.

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