Journal of Medical Sciences (2009); 2(3): 98-107

Review Article

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Unraveling the Molecular Basis of Alzheimer's Disease Yields Novel Therapies: A Personal Retrospective

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Among the myriad disorders that can devastate mental function, the example of Alzheimer's disease looms particularly large for neuroscientists. In part, this is because it is the most common of the brain degenerative diseases. But it is also because it begins with a remarkably pure impairment of cognitive function. Patients with Alzheimer's disease are robbed insidiously of their most human qualities: memory, reasoning, abstraction, insight and language. In the beginning, otherwise healthy individuals experience a subtle and intermittent impairment of their ability to encode new memories, first of trivial and later important episodes of everyday life. The gradual dissolution of the ability to register new details and then retrieve them when needed evolves in an individual whose motor and sensory functions are well preserved. Over a span of many months to several years, first declarative and then non-declarative memory are eroded, and other complex mental functions such as judgment and planning slowly slip away. But the subtlety of the earliest amnestic symptoms, occurring without clinical evidence of other neural dysfunction, suggest that something is discretely, perhaps intermittently, interrupting the function of synapses that encode new declarative memories. Research conducted over more than two decades suggests that this "something" is the amyloid β-protein ***(Aβ),*** a small, hydrophobic peptide with an ominous tendency to assemble into long-lived polymers in regions of the brain that serve memory and cognition. This chapter provides a personal retrospective of work in the author's laboratory that contributed to this conclusion.

In the decades after Alois Alzheimer, a Bavarian psychiatrist working first in Frankfurt and then in Munich, described his index case,[1] both the clinical and the neuropathological features of the disease were described in great detail. The disorder presented as a slow progression of cognitive symptoms, usually beginning with subtle impairment of recent episodic memory and proceeding to more severe memory difficulty, disorientation, confusion about details of everyday life, word-finding difficulty, problems with mathematical and geographical concepts, and often a lack of emotional homeostasis. Neuropathologically, individuals who died with this syndrome after 5, 10 or more years of devastating symptoms showed the two classical brain lesions Alzheimer had remarked upon: the neurofibrillary tangles and the senile (neuritic) plaques. These occur in large numbers in the limbic and association cortices of the brain and in certain subcortical neuronal populations (e.g., the basal forebrain cholinergic neurons) that project their axons to the cortex.

There were three possible approaches to the question of how to decipher the cause and mechanism of this pathology. One was to homogenize regions of the patient's brain and analyze them neurochemically, in order to

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learn which normal constituents were changed in amount and whether such altered constituents had a discernable link to mental function. This avenue was pursued effectively by searching for changes in neurotransmitters. By the mid-1970s, a few scientists had discovered that the hippocampus and cerebral cortex showed a profound decrease in the enzymes which synthesize and degrade acetylcholine (choline acetyltransferase and acetyl cholinesterase). This line of research indicated that neurons which use acetylcholine as a neurotransmitter die prematurely in the disease, but it did not reveal what might be killing them. Another potential approach would be to identify genes that caused inherited forms of Alzheimer's disease. It had been known for some time that certain families had an Alzheimer genetic trait that was transmitted in an autosomal dominant fashion. But methods to conduct rigorous genetic linkage analyses and subsequent positional cloning were only beginning to emerge in the 1970's, and large collections of Alzheimer families with multiple affected members were not generally available. A third approach, beyond the neurochemical and the genetic, was to pursue what is best described as biochemical pathology, meaning the attempt to analyze the protein composition, and ultimately the molecular origin, of the classical pathological lesions that define the disease.

The avenue of biochemical pathology was certainly not without its risks. Some authorities in Alzheimer's disease emphasized that the plaques and tangles were likely to be "tombstones" of a very long process and would not lead readily to an accurate understanding of the earliest events in the disorder. Although this was certainly possible, it was more likely that the innumerable neuritic plaques and neurofibril1ary tangles found in brain regions important for memory and intellectual function represented biochemical changes that were relatively close to the underlying molecular causes. It was this thinking that encouraged the isolation of the first neurofibrillary tangles and later amyloid plaques from a sizeable number of postmortem brains obtained with the invaluable collaboration of the spouses and children of Alzheimer victims.

The human neurofibrillary tangle-bearing neurons that were isolated from AD cerebral cortex were characterized using methods modified somewhat from those Shelanski had originally suggested.[2] When many such neuronal fractions were analyzed by denaturing gel electrophoresis to identify the subunit protein of the paired helical filaments (PHF) known form electron microscopy to comprise the tangles, no differences could be discerned in the protein composition of tangle-rich neuronal fractions versus identically prepared fractions from age-matched normal cortex devoid of tangles[3]. Despite the presence of abundant tangles, it appeared that their subunit protein could not be visualized on SDS polyacrylamide gels. In particular, an earlier report, that the subunit of the tangles was a ~50,000 MW SDS-soluble protein, could not be confirmed. A possible explanation for the inability to detect their constituent proteins was that the tangles were highly insoluble and thus not depolymerized by the denaturing agents [sodium dodecyl sulfate (SDS) and β-mercaptoethanol (βME)] used in protein gel electrophoresis.

Based on this hunch, many hours were spent by the author examining PHF-rich fractions from AD cortex under the electron microscope, both before and after their extraction in the SDS-βME denaturing buffer. The PHF of the tangles were still present after boiling the samples in the denaturants, and the PHF then appeared considerably enriched and "cleaner" than in a fraction prior to SDS-βME treatment. We established that most neurofibrillary tangles in AD brain were, in fact, resistant to these harsh solvents and remained behind as insoluble polymers. It was then clear why it had been difficult to identify specific protein differences between the PHF-rich and PHF-free brain fractions. Selkoe, *et al.*[3] thus provided perhaps the first example of the remarkable insolubility, and thus stability, that pathological aggregates of proteins have in neurodegenerative diseases. Thereafter, Ihara, *et al.* pursued our collaborative studies of

the unusual chemical properties of neurofibrillary tangles. We developed the first antibodies to such tangles and showed that they did not react with numerous normal brain proteins we probed.[4] This result suggested that the tangles were composed of conformationally altered epitopes of some unidentified brain protein. Only in 1985-86 was it recognized that the anti-tangle antibodies reacted with altered forms of the microtubule-associated neuronal protein, tau.[5] This result and closely similar observations from other laboratories, including Ihara's[6,7] and Khalid Iqbal's,[8-10] clearly indicated that tau was the major antigenic component of the paired helical filaments making up the tangles. Many subsequent biochemical analyses in numerous laboratories have confirmed that neurofibrillary tangles are largely, if not solely, composed of insoluble forms of the normally soluble tau protein.

This early work on the biochemical pathology of the neurofibrillary tangle helped to establish appreciate the remarkable chemical alterations of normal brain proteins that occur during neurodegeneration. Years later, it became apparent that other age-related brain disorders such as Huntington's, Parkinson's and amyotrophic lateral sclerosis were also characterized in part by the insolubilization and progressive accumulation of normally soluble neuronal proteins. Indeed, one of the most exciting lessons that has emerged from the study of these previously obscure diseases is that they share aspects of a general mechanism of protein misfolding and aggregation.

By 1983, it had become increasingly clear from the literature that tangles, while a hallmark of Alzheimer-type brain pathology, were by no means unique to this disorder. Indeed, tangles occurred in a wide range of etiologically distinct neurodegenerative disorders, including, for example, subacute sclerosing panencephalitis (SSPE, a very rare post-measles neurodegeneration) and Kuf's disease (a lipid storage disorder). This knowledge implied that neurofibrillary tangles could certainly be responsible for injuring neurons but they might arise from distinct insults. It was assumed by the author that during the course of Alzheimer's disease, an insult arose that was distinct from the causes of the other tangle-bearing syndromes, such as SSPE and Kuf's disease. Based on this reasoning, I concluded that the amyloid plaques of Alzheimer's disease might be a more specific - and perhaps earlier - feature of the disorder than were the tangles. In humans, amyloid plaques were known to accumulate primarily in Alzheimer's disease and Down's syndrome. Patients with trisomy 21 (Down's syndrome) invariably develop both the neuritic plaques and neurofibrillary tangles of Alzheimer's disease, but they generally do so already in their 30's and 40's, and this can be accompanied by further intellectual deterioration in these individuals, who are variably retarded in cognitive development from birth. The only other circumstance in which substantial numbers of cortical amyloid plaques occur is very late in life in many neurologically "normal" humans and lower primates. Based on this understanding, methods were developed for isolating and purifying the amyloid that made up the cores of the innumerable senile plaques found in limbic and association cortices of AD brains. In the author's lab we found that the amyloid plaques, like the tangles studied earlier, were highly insoluble and remained largely intact after treatment in harsh denaturants such as SDS and guanidine hydrochloride. Their insolubility and their spherical shape were taken advantage of to use fluorescent-activated cell sorting to purify them from other brain constituents, as if they were small, round lymphocytes (whose dimensions they resembled). George Glenner and Caine Wong took the approach of isolating not the amyloid plaques but rather the meningovascular amyloid deposits that occur in most Alzheimer brains.[11,12] Glenner, who had worked successfully for many years on systemic human amyloidoses, isolated the subunit protein of the vascular amyloid, which he named the ***β*-**protein [now referred to as amyloid β-protein ***(Aβ)].*** Glenner's isolation of A*β* in 1984 has turned out to be the seminal event in the un-

raveling of the pathogenesis of Alzheimer's disease, in my view.

Amino acid analyses we performed indicated that the composition of the purified plaque cores was very similar to that which Glenner reported for the meningovascular amyloid subunit. In 1985, Colin Masters and co-workers isolated and biochemically analyzed plaque core fractions. They came to a similar conclusion: amyloid plaque cores, which they solubilized in 70% formic acid, yielded a protein fragment that was similar if not identical to the β-protein Glenner had described.[13] The purification method of Selkoe *et al.* was published shortly after theirs, together with further biochemical and immunochemical characterization of the highly purified amyloid plaque cores.[14] Next, we examined the biophysical characteristics of the purified amyloid plaques by X-ray fiber diffraction analyses. These studies revealed a cross β-pleated sheet conformation typical of classical amyloid deposits in systemic organs.[15] Shortly thereafter, Kirschner, *et al.* showed that synthetic peptides made to the partial sequence of *Aβ* could likewise assume stable, cross β-pleated sheet conformations, providing an initial *in vitro* model of the Alzheimer amyloid fibril.[16]

By the time the analyses of both natural, purified amyloid plaque cores and synthetic Aβ assemblies were published, it had become clear that the accumulation of the amyloid protein as an insoluble aggregate represented a reasonable candidate for the etiologic agent of Alzheimer's disease. In other words, the characteristics that Glenner, Masters, Selkoe and others were describing for the cerebral amyloid of Alzheimer's disease were highly reminiscent of those found in various systemic amyloid deposits that can be causative of distinct human amyloidoses. Because some of these disorders were known to be caused by mutations in the genes encoding the respective amyloid subunit proteins, it was reasonable to believe that both familial and sporadic forms of Alzheimer's disease could be initiated by accumulation of the amyloid β-protein in either mutant or wild-type forms, respectively.

In early 1987, the identification by four laboratories of the gene encoding the precursor protein of Aβ (the amyloid β-protein precursor, or APP) led to several exciting insights. One that was particularly intriguing was the fact that the ~40 amino acid Aβ peptide was a fragment of APP predicted to derive in part from the single transmembrane domain of this receptor-like molecule (*Fig. 1*). This was highly curious, because it suggested that one of the two putative proteolytic cleavages that released Aβ from its precursor must occur within the transmembrane region of the molecule. However, there was no known mechanism by which a proteolytic cleavage within the membrane could occur, as hydrolysis of a peptide bond requires water molecules, and these would not be found in the hydrophobic environment of a lipid membrane. This apparent conundrum would occupy much attention in the next decade and a half.

The other exciting insight that emerged from the cloning of APP was that its gene was found on the long arm of human chromosome 21. This recognition provided an immediate (and as it turns out, correct) hypothesis for why patients with trisomy 21 invariably develop Alzheimer-type pathology early in life. Some years later, it was found that early Aβ deposits (so-called "diffuse" or "pre-amyloid" plaques) could be found in large numbers in the cerebral cortex of Down's patients as early as age 12, long before mature, amyloid-bearing neuritic plaques and neurofibrillary tangles arose. This work in our lab and similar studies in other labs strongly suggested that accumulation of Aβ in such early deposits preceded the development of other neuropathological features of AD by years or decades, at least in patients with trisomy 21.

The first biochemical identification of the heterogeneous polypeptides that comprise the APP gene products in the brain was provided by our lab when we noted that APP underwent a proteolytic cleavage to yield a ~ 10 kDa C-

sible to study the conversion of APP to Aβ dynamically in cultured cells and in animals. Prior to this, Aβ had only been observed after painstaking purification of insoluble amyloid deposits from postmortem human brain tissue. But now, it was clear that essentially any cultured cell could be analyzed for its secretion of *Aβ.* Second, the presence of AB in normal biological fluids, such as cerebrospinal fluid, plasma and the conditioned medium of biopsied skin fibroblasts, suggested that one should be able to quantify Aβ and follow changes in its amount over time and during the treatment of Alzheimer's disease. Indeed, the measurement of Aβ42 in CSF has subsequently been shown to be useful as a biomarker of Alzheimer's disease. Third, and perhaps most important, the finding of normal Aβ secretion provided cell culture assay systems that could be used to screen large libraries of chemical compounds in order to find those that could lower Aβ production without injuring the cells.

Each of these three implications of the discovery of soluble Aβ has been extensively exploited in the years since the initial reports. An APP mutation discovered by geneticists Michael Mullan and Lars Lannfelt to cause a rare form of early-onset AD in a Swedish family[23] increased the cellular production of Aβ about 5-8 fold.[24] This observation and a similar finding from Steve Younkin and colleagues provided the first genotype-to-phenotype relationship in familial Alzheimer's disease. The mechanisms of other APP mutations, including in skin fibroblasts cultured directly from affected patients, were subsequently examined.[25] After presenilin (PS) was discovered as an AD-causing gene by Peter St. George Hyslop in 1995,[26] we and others analyzed the effects of various pathogenic mutations in PS1 and PS2 on APP processing. In all cases, it was found that genetic mutations causing early-onset familial AD led to an increase in the production of *Aβ,* but in particular, the hydrophobic and highly self-aggregating Aβ42 species. This evidence for a simple, direct conversion of Alzheimer-causing genotypes to the phenotype of cerebral Aβ accumulation remains one of the strongest supports for the "amyloid" hypothesis (now more correctly the Aβ hypothesis) of Alzheimer's disease.

Among the rewarding aspects of studying the mechanisms of a disease are the implications one's discoveries may have for fundamental biology. This has been well-illustrated by the research on the presenilin protein and its connection to the unidentified protease referred to as γ-secretase. The finding of normal Aβ production mentioned above suggested that two proteolytic activities, nicknamed β-secretase and γ-secretase in the field, must cleave APP at the N- and C-termini of the Aβ region, respectively, to liberate small peptides of 39-43 amino acids into intraluminal and extracellular fluids. Because mutations in presenilin cause the most aggressive known form of AD by increasing the generation of Aβ42 peptide compared to that of the Aβ40 peptide, it appeared that presenilin could modify the γ-secretase reaction in some way. In other words, presenilin mutations allowed γ-secretase to cleave more APP molecules after the 42nd rather than the 40th residue in the Aβ sequence, leading to more Aβ42. Peter Lansbury and colleagues had previously shown that Aβ42 had a particularly strong tendency to seed amyloid fibril formation.[27] To approach how presenilin alters γ-secretase processing of APP, evidence was obtained that small fractions of APP and PS1 could be co-immunoprecipitated from cells, even at endogenous levels of protein expression.[28] Various subcellular fractionation experiments were performed that convinced us that APP and presenilin were often found in the same vesicular fractions of cells, and they could again be co-precipitated from such vesicles. Indeed, presenilin and APP contacted each other within subcellular vesicles in which the *de novo* production of AB upon incubation at 37°C was demonstrated.

Michael Wolfe decided to design and synthesize peptidomimetic inhibitors of γ-secretase that mimicked the Aβ40-45 region within APP. By installing difluoro ketone or difluoro alcohol moieties in the PI/PI' positions of these substrate-based molecules, he showed that they inhib-

ited cellular Aβ production, we concluded that whatever γ-secretase was, it was likely to be an aspartyl protease rather than a member of one off the three other classes of protein-cleaving enzymes.[29]

Bart DeStrooper and coworkers[30] reported the striking observation that deleting the presenilin 1 gene in a mouse caused a sharp decrease (~70%) in Aβ production by its neurons. Although this finding was generally interpreted to suggest that PS served as a critical cofactor for the unknown γ-secretase, Wolfe and I was felt that another plausible interpretation was that presenilin was the actual protease. Much of the combined evidence obtained thus for (above) was consistent with this provocative hypothesis, and we pursued this. A close inspection of the presenilin amino acid sequence led Wolfe *et al.*[29] to observe two aspartate residues in adjacent transmembrane domains of both presenilin 1 and presenilin 2 (the two human homologues). This recognition led immediately to our speculating that the two aspartates might serve as the active site of an unprecedented intramembrane aspartyl protease. Subsequent mutagenesis of the aspartates in PS1 led to a dramatic drop in cellular Aβ production, to about the same extent as knocking out the entire PS1 gene. Moreover, mutating either intramembrane aspartate abrogated the normal endoproteolysis of presenilin into its biologically active heterodimeric form. These results were interpreted by us to indicate that the two aspartates were the active site of γ-secretase and could activate the enzyme by autoproteolysis.[29] It was of great interest that all homologues of presenilin in lower animals (e.g., in C. *elegans* and *Drosophila)* also contained these two aspartates. Upon our publication of these data in early 1999, several laboratories confirmed and extended the findings, showing that the aspartate mutations acted in a dominant-negative fashion to markedly alter the function of presenilin in intact cells. Nonetheless, the hypothesis that presenilin was the actual γ-secretase remained controversial.

It had become apparent from the work of Iva Greenwald,[31] Raphael Kopan,[32] Bart DeStrooper[33] and others that this unusual protease had a crucial role in the normal development of all multicellular organisms - as a mediator of the Notch signaling pathway. Work in these laboratories showed that *C. elegans*, *Drosophila* and mammals all required their presenilin homologues for proper function of Notch. More specifically, a cleavage within the Notch transmembrane domain first described by Kopan and colleagues, liberated the Notch cytoplasmic domain to signal in the nucleus, and presenilins were shown to be required for this processing. It was found that the aspartate mutations in PS1, as well as the APP substrate-based γ-secretase inhibitors mentioned earlier, could markedly interfere with Notch nuclear entry and thus decrease Notch signaling. This line of research meant that PS was necessary not only for generating the Aβ peptide of Alzheimer's disease, but also for the proper function of Notch in cell fate decisions during the development of all metazoans. If γ-secretase (aka presenilin) was inhibited with such compounds there was a risk of interfering with the normal function of Notch. Importantly, this function is not restricted to early development; it is required for cell fate decisions throughout adulthood, such as during hematopoeisis and the turnover of epithelial cells. Fortunately, recent studies by several laboratories have identified a variety of small molecules that can modulate presenilin/γ-secretase in a ways that lower the cellular production of Aβ42 but only interfere importantly with Notch processing at much higher concentrations.

In the last few years, three additional proteins that are required for the function of presenilin in the processing of APP, Notch and a growing number of single-transmembrane substrates have been discovered by several laboratories, using either biochemical purification or genetic screens in invertebrates. These apparent cofactors are Nicastrin, Aph-I and Pen-2. My collaborators and I built on these discoveries to show that only the co-expression of all four pro-

oligomers of AB might be responsible for some of the alterations in hippocampal synaptic activity that presumably underlie memory impairment in AD patients.

Finally, it is intriguing that there are rapidly emerging parallels between the work on the pathobiology of AD summarized above and the attempt to implicate soluble oligomers of misfolded proteins in the mechanisms of other neurodegenerative diseases. Indeed, this process as regards α-synuclein in Parkinson's disease has begun to be studied. Years of research will be needed before we will learn whether α-synuclein accumulation has a similar import for the development of idiopathic Parkinson's disease that an imbalance between Aβ production and clearance does for Alzheimer's disease.

The application of molecular and cell biological methods to the elucidation of Alzheimer's disease, with special attention to inherited forms of the disorder, has allowed us to develop and refine a hypothetical model describing how the disease begins, unfolds and eventually produces progressive dementia (*Fig. 2*). This schema remains a hypothesis, though it is one that has strong experimental support but that can ultimately be validated only in the clinic. The ideas and findings reviewed in this article have helped bring us into a time of human trials of Aβ-lowering therapeutic agents. If one or more of these is shown to delay the development or slow the course of progressive cognitive failure, then Alzheimer's disease may turn out to exemplify the power of reductionist science applied to the most complex of biological systems, the human cerebral cortex.

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