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Translating Discoveries in Basic Molecular Biology, Cell Biology, and Molecular Genetics into Transformative Approaches to the Diagnosis and Treatment of Currently Incurable Neurodegenerative Dementias

PETER ST GEORGE-HYSLOP

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Short Biography

Peter Henry St George-Hyslop, MD, FRS, FRSC, FRCPC, (born July 10, 1953) is a British and Canadian medical scientist, neurologist and molecular geneticist who is known for his research into neurodegenerative diseases.

He has identified a number of key genes that are responsible for nerve cell degeneration and early-onset forms of Alzheimer's disease.

Since 2007 St George-Hyslop has headed an Alzheimer's disease research program as Professor of Experimental Neuroscience at the University of Cambridge. Educated at Wellington School, Wellington, Somerset, UK, St George-Hyslop completed his medical training in Canada, graduating with the MD degree in 1976, and then pursuing post-doctoral research in internal medicine and neurology at the University of Toronto and Harvard Medical School.

He served his first appointment at Harvard's Massachusetts General Hospital, where he taught molecular genetics and neurology from 1987 to 1991.

He was appointed to the University of Toronto in 1991, and since 2003 has held the university's highest rank of University Professor. Since 1995, St George-Hyslop has served as the director of the Tanz Centre for Research in Neurodegenerative Diseases at the University of Toronto Faculty of Medicine.

In 2007 St George-Hyslop was appointed Professor of Experimental Neuroscience at the University of Cambridge.

His studies focuses upon understanding the causes and molecular mechanisms of neurodegenerative diseases such as Alzheimer's Disease,

Parkinson's Disease and Fronto-Temporal Dementia. St George-Hyslop has shown that these diseases are frequently caused by the accumulation neurotoxic proteins or protein fragments. He and his team employ genetic, molecular biological, cell biological, and animal modeling strategies to: 1) identify disease-causing genes; and 2) to identify the molecular pathways by which these mutation or polymorphisms lead to neuronal death.

He was awarded the Howard Hughes Medical Institute International Scholar Award in 1997 and 2002, the Gold Medal in Medicine from the Royal College of Physicians of Canada in 1994 and the Michael Smith Award from the Canadian Institutes of Health Research in 1997. He is a member of the American Society for Clinical Investigation, a fellow of the Royal Society of Canada, and a Foreign Member to the Institute of Medicine of the United States National Academies.

Contents

Summary

Neurodegenerative diseases, and Alzheimer's disease (AD) in particular, represent an emerging worldwide health crisis. In the next forty years, the numbers of humans affected with these late life disorders will more than triple. Unless effective therapies are found, this anticipated deluge of patients will exceed the capacity of most societies to humanely care for them. However, the current manuscript describes how recent scientific work, led in no small part by the author, has begun to transform our understanding of these diseases. For the first time, knowledge emerging from basic science is being used as a rational basis to create clinically-useful diagnostics and therapeutics.

Until about 20 years ago, these disorders were considered abstruse and rare illnesses. Little was known beyond the clinical descriptions of patients' behavior and symptoms, and the observations made on microscopic sections of postmortem brain. However, in the past 20 years, groundbreaking work has been pursued by the author and colleagues in the field using cutting-edge tools in molecular genetics, molecular and cellular biology, structural biology, and animal modeling. The emerging results have radically changed our understanding of these diseases. Some of the greatest epiphanies have arisen from the identification of genes causing these disorders - an approach to neurodegenerative dementias that was pioneered by the author beginning in the 1980s. The genes causing neurodegenerative dementias and the proteins that they encode have been characterized by the author and colleagues using biochemical, molecular, cellular invertebrate and vertebrate animal systems. Together, these studies have driven monumental gains in our understanding of the molecular mechanisms of these diseases.

In AD, at least 15 different genes have been identified that are associated with increased risk for AD. Several of these genes (e.g. APP, PS1, SORL1) impact the processing of the APP gene and lead to the production of neurotoxic forms of a proteolytic peptide fragment termed Aβ. Several other genes also affect risk for AD. Many appear to be involved in mediating the downstream consequences of the accumulation of Aβ aggregates. Some of these genes are involved in innate immune and inflammatory responses to the presence of aggregated proteins (e.g. CR1, CLU). Others are possibly involved in the uptake and inter-cellular spreading of protein aggregates (e.g. BIN1, PICALM). Other downstream consequences include misprocessing of tau.

In Fronto-Temporal Lobar Degenerations/Motor Neuron Disease spectrum (FTLD/MND) the genetic studies suggest that there are multiple independent pathways that can cause the same clinical phenotypes. Some of these pathways involve misprocessing of the tau protein in neurons or glia. In other subtypes of FTLD, the genes are either members of a class of hydrogel-forming RNA binding proteins (e.g. FUS and TDP-43), or they are genes that modulate the processing of these proteins (e.g. GRN and C9ORF72). When mutant or misprocessed, FUS and TDP43 form neurotoxic aggregates in the cytoplasm of neurons.

The knowledge emanating from cutting-edge basic science research has begun to significantly change clinical practice. Knowledge of the underlying molecular events has formed the basis for biomarkers with which to diagnose these diseases. These biomarkers include measurements of disease-causing proteins and protein fragments in the CSF. They also include neuroimaging methods for measuring the accumulation of these proteins in the brain, or the activation of inflammatory pathways.

The disease-associated genetic variants can themselves be used clinically. One such use has been to permit the longitudinal follow-up of pre-symptomatic carriers of disease-causing mutations. This has led to the discovery, by the author and colleagues, that neurodegenerative dementias typically have long pre-symptomatic periods (up to 15 years). This discovery has two critical implications. First, it presents an unprecedented opportunity to detect and treat at-risk individuals long before permanent brain damage has occurred. Second, epidemiological studies reveal that if this pre-symptomatic phase could be extended by only five years, it would result in a 50% reduction in the number of cases. Genetic tests can also be used clinically for: genetic counseling of asymptomatic family members; for clarification of the diagnosis and prognostication in symptomatic cases; and for the creation of more homogeneous genotypically-focused cohorts of patients for clinical trials. This latter use of genotype information is likely to transform Experimental Medicine approaches to the testing of new medicines. Genotype data will also be used in Personalised Medicine approaches where a patient's therapy is tailored to the underlying genetic cause(s) of their disease.

Finally, the increasing knowledge about the underlying molecular mechanisms has activated attempts to identify tractable therapeutic targets within

these molecular pathways. The most recent attempts to target the disease mechanisms in AD have focused on blocking the accumulation and aggregation of Aβ. The preliminary experiences with experimental anti-Aβ therapies have been frustrated by: 1) problems of inadequate access of the experimental therapy across the blood brain barrier; 2) drug-induced toxicity; and 3) flawed design of the clinical trials where target engagement was not pre-documented, or where the clinical trial was performed on patients with advanced disease. Nevertheless, post-hoc analysis of the data from some of the better designed trials (e.g. the Phase 3 trial of Solanezumab anti-Aβ antibody) provides a glimmer of hope. Subjects with mild disease appear to show an improvement.

In summary, in 20 years, the field has progressed dramatically from knowing very little, to now being on the cusp of having effective diagnostics and therapeutics. The author has played a central role in this explosion of knowledge. He has played key roles in the discovery of genes associated with AD, PSP, and FTLD. He has helped characterize the products of these genes, and the effect of mutations. He has generated tractable cellular and animal models of these disorders. He has generated proof-of-principal studies for several candidate therapies, two of which have gone into Phase II or Phase III clinical trials in humans. This body of work is described in detail in the following paragraphs.

I
Introduction

Neurodegenerative dementias, and Alzheimer's disease (AD) in particular, are emerging as leading worldwide public health problems. 35.6 million people were estimated to be living with dementia in 2010. There are 7.7 million new cases of dementia each year. As the number of persons over 65 years of age increases, the prevalence will triple over the next 40 years[1-3]. This dramatic rise in prevalence has immense personal and economic implications for all societies (Figure 1). The costs are estimated at US$604 billion per year at present and will increase even more quickly than the prevalence. Currently 58% of people with dementia live in low and middle income countries and this prevalence is projected to increase to 71% by 2050 in China, India, and Latin America. By 2050, people aged 60 or older will account for 22% of the world's population, four fifths of whom will be living in Africa, Asia, and Latin America (http://www.who.int/mental_health/publications/dementia_report_2012/en/). Consequently, in many emerging countries, the predicted increasing prevalence of dementia and dementing disorders will outstrip the ability of low- and middle-income nations to effectively manage patients with these diseases.

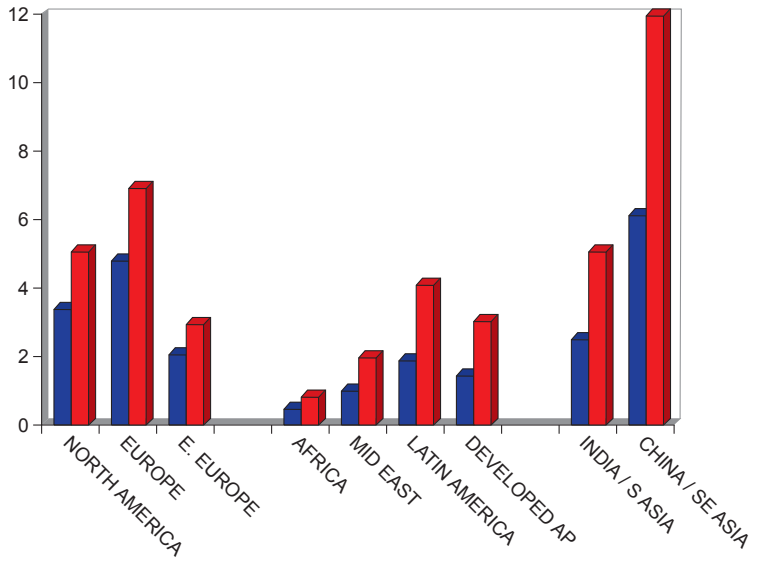


Figure 1: Anticipated worldwide increase in dementia cases (in millions) between 2001 (blue) and 2020 (red).

Until about twenty years ago, neurodegenerative diseases such as Alzheimer Disease and Frontotemporal dementia were abstruse diseases, largely the domain of dusty neuropathology textbooks. Little was known beyond the phenomena that could be observed from the patients' behaviour and from the stained microscopic neuropathology slides of post-mortem brain tissue (summarized in Table 1). Until 20 years ago, our understanding of these diseases had essentially changed very little since the studies of Alois Alzheimer, Pick and others. However, in the past 20 years or so, there has been a veritable explosion of knowledge. The present review describes how critical discoveries in fundamental biological research made over the last 20 years by the author and his colleague scientists have dramatically transformed our understanding of these diseases. This truly massive revolution in our understanding, which emanates from the application of structural biology, molecular biology, cell biology, molecular genetics and vertebrate and invertebrate animal modeling, has provided an unparalleled opportunity for the rational development of new tools to diagnose and treat these disorders.

Presently there are no proven disease-modifying treatments or preventions for dementia. However, based on the recent transformative discoveries in basic molecular, cellular and genetic science that will be described below, there is now great hope. Indeed, several candidate disease-modifying therapies for various neurodegenerative dementias are under investigation. For example, in AD, there are now many newly-emerging disease-modifying therapies that are intended to block neuronal injury caused by Aβ aggregation. Disease modifying therapies are also being developed against other features of AD (e.g. tau aggregation and/or inflammation). For some forms of Frontotemporal Lobar Degeneration (FTLD) that are caused by mutations in progranulin, the use of drugs to increase progranulin transcription shows great promise.

However, while there is increasing optimism that effective disease-modifying therapies may be on the horizon for at least some forms of dementia, there is increasing belief that the best opportunity for successful therapeutic intervention in these diseases will be via treatment during the pre-symptomatic phase. This concept is based upon recently emerging evidence from biomarker studies, which suggest that, with the exception of prion-related dementias, patients with neurodegenerative dementias nearly always have a

long pre-symptomatic period. In this preclinical phase, biochemical, neuropathological and neuroimaging changes can be detected by at least a decade before the onset of clinical features4,5. Clearly, if sensitive but cost-effective neuroimaging or biochemical screening tools (biomarkers) can be implemented, it might be possible to routinely screen and identify patients for preventative treatments. Equally importantly, because of the late age of onset of AD, a treatment in this pre-symptomatic period that caused even just a five-year delay in onset of symptoms would result in a 50% reduction in the number of cases[1].

Diagnostic and therapeutic successes along these lines are on the horizon. Their development is based on the huge and unprecedented advances in our understanding of the molecular mechanisms underlying these diseases that have occurred in the past 20 years. The paragraphs below depict the basic science tools that have been used (Section and then specific knowledge about the molecular pathways that has arisen (Section III). The final section then describes how this knowledge is being translated into practical clinical benefits for patients (Section IV).

Table 1. Key Clinical Features of the Diseases Described Here

Disease	Key Features
Alzheimer's disease	Slowly progressive (10 years). Loss of parietal and temporal functions with impaired memory agnosias, apraxias and language.
Frontotemporal Lobar Dementia	Slowly progressive (10 years). Early loss of frontal and temporal functions with prominent social and behavioural deficits, impaired motor speech. Memory and parietal functions affected late. May have ALS-like features.
Familial British/Danish Dementia	Slowly progressive (10 years) with predominant loss of parietal and temporal functions. Early spasticity and ataxia in Familial British dementia. Early posterior cataracts, ataxia, hearing loss in Familial Danish dementia. Onset 20 -50 years.
Familial Encephalopathy with Neuroserpin Inclusion Bodies	Slowly progressive (10 years) with predominant frontal features. Memory affected late. Onset before 65 years. 5-50 micron neuronal inclusions that are PAS- positive, diastase resistant (Collins Bodies).
Creutzfeldt-Jakob Disease	Rapidly progressive (<1-2 years). Dementia often accompanied by myoclonus. Variants include: 1) Gerstmaan-Straussler (progressive ataxia, dysarthria, with cognitive decline occurring only late); 2) Fatal Familial insomnia (progressive insomnia, dysautonomia and myoclonus); and 3) variant CJD (associated with psychosis and puritus in young adults exposed to BSE).

II
Basic Science Approaches To Neurodegenerative Dementias

The author and other scientists working on neurodegenerative diseases have used two general approaches to very successfully dissect the molecular mechanisms underlying neurodegenerative dementing diseases.

One paradigm has employed molecular biology, biochemistry, neurophysiology and cell biology to address specific hypotheses about the nature and effects of protein aggregates observed in these diseases.

The other paradigm has used a hypothesis-free approach by applying cutting-edge recombinant DNA gene mapping methods to identify the genes causing susceptibility to these diseases, and then work forward from the biology of those disease causing genes.

As is described in the next few pages, both approaches have been dramatically productive, and often in powerfully complementary ways. This manuscript will therefore describe both approaches, but will focus especially on the genetic approaches for the three following reasons.

First, by virtue of its hypothesis-free nature, the genetic approach has repeatedly generated discoveries that have literally been unexpected paradigm-shifting "game-changers". These discoveries have massively impacted both our understanding of these diseases and also our understanding of fundamental biology in general. For instance, the discovery of the presenilin genes by the author[6] (see below), led not only to a better understanding of the biology of Alzheimer's disease, but also led to the discovery of a hitherto undescribed biological process, in which cell surface receptors are proteolytically cleaved in situ within cellular membranes. The cleavage transects the transmembrane domains of the substrate. This latter process, termed "Regulated Intramembranous Proteolysis" by the Nobel Laureate J. Goldstein[7], turns out to be necessary for a number of biologically-important inter-cellular signaling events that are essential for life during development and postnatal life (e.g. Notch-Delta signaling during embryogenesis).

Second, the genetic discoveries have typically been discoveries that could never have been made using a hypothesis-dependent approach. For instance, at the time they were cloned for the first time by the author[6], the presenilin proteins had never been encountered before. Their gene sequences were completely unknown to biology. It was only after the presenilins were first identified by the author using a genetic approach[6] that it became clear from work by the author[8] and others (see below), that the presenilins func-

tioned as the enzyme which produced Aβ by cleavage of the amyloid precursor protein (APP).

Third, the genetic approach has generated discoveries that relate ***directly*** to the cause and mechanism of the disease. Thus, when disease-associated mutations are found in a gene, it means that defects in that gene are the direct and proximate cause of the disease. In contrast, most of the molecular biological discoveries have been ambiguous in terms of their role in the disease pathogenesis. For instance, following the discovery of the amino acid sequences of the Aβ and tau protein deposits in neurons of patients with AD, it remained a very unclear for several years as to whether the accumulation of Aβ and tau were causing AD, or were simply innocent and secondary effects of the disease ("tombstones"). The unclear role of Aβ and tau was dramatically and definitively resolved by genetic studies. These genetic studies unambiguously demonstrated that mutations in APP/Aβ and tau genes ***caused*** neurodegenerative disease.

II.1. Basic Methods for Molecular Biological Approaches in Dementia Research

The early neuropathological studies in AD dating back to the original descriptions by Alois Alzheimer[9,10] had identified the presence of amyloid plaques and neurofibrillary tangles as robust features of the disease (see Figure 6 below). Similar neuropathological studies in FTLD also led to the discovery of abnormal intraneuronal inclusions composed of ubiquitinated aggregated proteins. However, there was no indication from these early clinical and neuropathological studies as to the biochemical nature, cellular origins or functional significance of any of these neuropathological features.

In the 1980s and 1990s, careful and difficult biochemical purifications and amino acid sequence analyses were used to identify the component proteins in amyloid plaques and neurofibrillary tangles. Biochemical analysis of proteins isolated from purified amyloid plaques and vascular amyloid deposits from AD patients led to the discovery of the amino acid sequence of what is now called Aβ peptide. Aβ was subsequently shown to be a proteolytic fragment of longer amyloid precursor protein (APP)[11] (see below). Similar

studies on proteins purified from neurofibrillary tangles provided the peptide sequence of what is now called the microtubule associated protein tau (or simply tau)[12,13]. An analogous approach led to the discovery of the protein sequences of TDP-43 protein in inclusions in neurons from some cases of FTLD[14].

Once the peptide sequences were identified, further molecular biological / molecular genetic work was required to identify the genes encoding these proteins. Thus, in AD, several groups including the author's[15], worked to clone the gene encoding the protein containing the Aβ peptide (which turned out to be the APP gene on chromosome 21[15-19]). Simultaneously, other groups worked to find the gene encoding the tau protein (which turned out to be the microtubule associated protein tau (MAPT or tau) gene on chromosome 17)[12,13]. The same paradigm was pursued for the mapping of the genes associated with intraneuronal deposits of TDP-43 and α-synuclein. These molecular biological studies often serendipitously coincided with molecular genetic studies that were focused on discovering susceptibility genes for these diseases. Thus, in AD, the cloning of the APP gene occurred concurrently with the mapping of a susceptibility gene for familial AD in the same region of chromosome 2115,19,20. The same convergence of events also held for one form of FTLD. The tau gene was mapped to chromosome 17[12,13]. Independently, genetic linkage studies identified a susceptibility gene for a specific subtype of FTLD (FTDP-17) in that same region of chromosome 17[21]. These convergences prompted nucleotide sequencing of the candidate gene, and the eventual discovery of the underlying disease-causing mutations within these genes.

The discovery of disease-causing missense mutations drove the next set of studies to understand the impact of the disease-causing mutations. Typically, this next-generation of molecular studies applied molecular and cell biological methods in cell-free biochemical systems, in transfected cells, and in invertebrate and vertebrate model organisms. This work in turn led first to the development of extensive background knowledge about the biology of the wild type (normal) protein, and then an understanding of how this normal function was changed by the presence of disease-causing mutations. For instance, in AD, a large amount of work over several years developed an understanding of the events surrounding the normal intracellular trafficking

of APP, and about its physiological post-translational proteolytic cleavage to generate Aβ peptide. Similar molecular and cell biological experiments led to an understanding of the normal biology of other disease-causing genes including tau, progranulin, TDP-43 and FUS.

Concurrently, biochemical and biophysical studies on these proteins demonstrated that disease-causing mutations in these proteins resulted in either their misfolding and aggregation (e.g. tau, TDP-43, FUS), or to misfolding and aggregation of proteolytic fragments of the mutant disease-associated protein (e.g. Aβ peptide fragment of mutant APP). Much additional work is still ongoing to understand the exact biophysical nature of these protein aggregates. Many of these aggregated neurodegeneration-associated proteins form classical amyloid fibrils. These classical amyloids stain with dyes such as Congo Red and Thioflavin S, and contain cross-β sheet structures on x-ray diffraction analysis. However, biophysical studies, including more recently single molecule analyses have revealed that the fibrillar and monomeric forms of these aggregation-prone proteins are in a metastable equilibrium with each other and with a large number of soluble intermediary species[22-35]. It now appears that a subset of these intermediary soluble oligomeric species are highly toxic to neurons (reviewed in)[36].

However, very recently, as described in detail below, the author and colleagues have discovered that classical amyloid proteins are not the only form of neurotoxic protein aggregate[37]. We have discovered the existence of a novel form of toxic protein aggregate that is derived from misfolding of hydrogel forming proteins like TDP-43 and FUS (manuscript under review)[37]. Hydrogel-forming proteins have the physiological capacity to transiently, and reversibly aggregate, for instance, during stress reactions. This physiological, reversible aggregation allows the hydrogel-forming protein to capture key RNA species and RNA binding proteins and hold them on stress granules, and to then release them again when the cellular stress is removed. The author and colleagues have now discovered that the disease-associated missense mutations in these hydrogel-forming proteins cause them to misfold. This misfolding converts the hydrogel protein from a reversibly aggregating, physiological and non-toxic state, into a state that is irreversibly aggregated and neurotoxic.

This recent observation by the author confirms the emerging concept that most, if not all, neurodegenerative dementias are due to the accumulation of neurotoxic protein aggregates. However, it broadens the types of aggregates that are neurotoxic.

Several approaches have been applied to understand how these aggregated protiens damage neurons. One of the most productive approaches has been to investigate the impact of these misfolded oligomeric species on the electrophysiology of synaptic transmission and synaptic plasticity. This has been particularly enlightening in AD, where it has been clearly shown that soluble oligomeric aggregates of Aβ damage synaptic structures, particularly the postsynaptic spine (Figures 2, 3). Indeed, alterations in synaptic function arising from the neurotoxic effects of aggregated oligomers of Aβ likely account for a significant proportion of the cognitive failure in Alzheimer's disease (AD)[38-48]. However, while the synaptotoxic effects of Aβ oligomers have been robustly replicated in cultured neurons, hippocampal slices, and transgenic mouse models, the underlying signaling mechanisms remain controversial[4,41-51]. Thus, there is no agreement on the precise identity of the neurotoxic oligomeric Aβ species (estimates ranging from dimers to dodecamers)[52-55], or on how these species induce neuronal damage. Hypothesis-driven experiments have suggested multiple candidate receptors for soluble Aβ oligomers including PrP^{C} [56], α-7 nicotinic receptors[43], RAGE receptors[57], insulin receptor-sensitive Aβ-binding protein[54], presynaptic P/Q calcium chan-

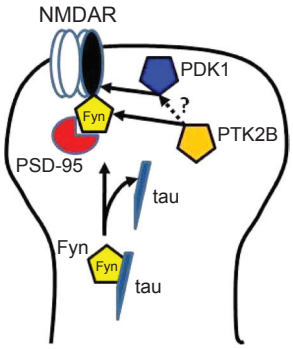


Figure 2: Tau transports Fyn kinase to the postsynaptic spine where it phosphorylates NR2B, and promotes interactions with PSD95.

nels[58], APP[59-61], sphingomyelinase[62], NMDA receptors, EPH2B receptors[63], membrane lipids[64], and the creation of ion permeable membrane channels[65]. The same type of hypothesis-driven analyses also suggested multiple different downstream signalling pathways. Under specific experimental circumstances caspase 3[66,67], caspase 2, protein phosphatase 2B (PP2B), tyrosine phosphatase STEP[43], GSK3beta[66], Fyn kinase[68], EPH2B[63], and changes in mitochondrial function and calcium metabolism have all been suggested to play important roles in the synaptic defects in AD (reviewed in refs[41,69]). However, when these candidate receptors and pathways have been blocked/inhibited, the effect has usually been modest. Consequently, while the work discussed above is likely to be correct under the specific hypothesis-driven experimental conditions in which these experiments were done, it is unclear which, if any, of the hypothetical mechanisms emanating from this prior work are truly relevant to the *in vivo* pathogenesis of AD. It is also far from clear that the postulated mechanisms are the <u>*only*</u> mechanisms by which Aβ and tau aggregates damage synapses.

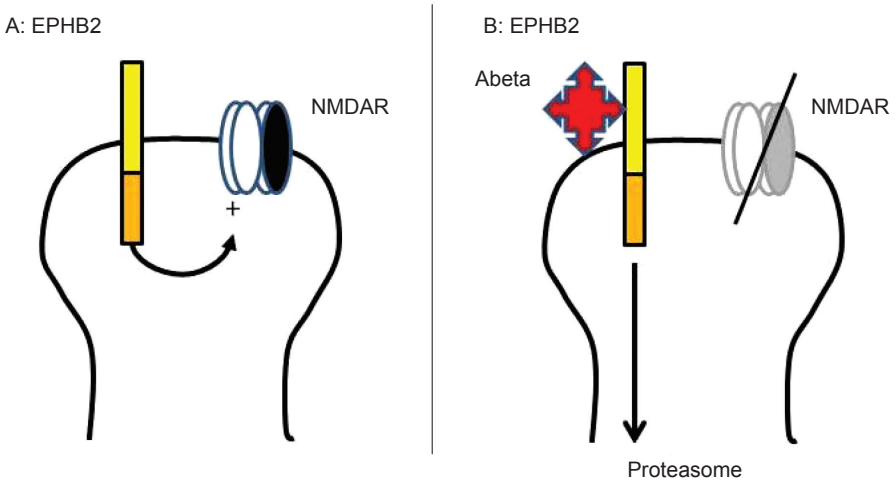


Figure 3: EPHB2 phosphorylates and stabilises NR2B NMDA receptor subunits. EPHB2 binds Abeta oligomers, and on endocytosis undergoes proteasome degradation, leading to destabilisation of NMDA receptors and the subsequent involution and removal of the postsynaptic spine.

Another approach to understand how aggregate proteins can injure neurons has been to investigate their effect on inflammatory cells in the brain (microglia and astrocytes). Indeed, this is an area of the cell biology of neurodegenerative diseases that has recently been profoundly influenced by genetic studies, to the point of inducing a complete reversal in thinking about its importance.

Until very recently, the presence of neuro-inflammation was well recognised, but was generally depicted as a late, secondary consequence arising from neuronal degeneration and cell rupture. However, as described in detail below, recent genetic studies by the author and colleagues has demonstrated that in fact inflammation is a key component early these diseases. Thus, in AD for instance, variants in genes like clusterin, complement receptor 1, CD2AP, and TREM2 (which are involved in innate immune and microglial inflammatory pathways) are all significantly transcriptionally dysregulated in AD brain and in the brain of transgenic mouse models. In addition, genetic sequence variants in these genes are strongly associated with risk for late onset "sporadic" forms of AD.

II.2. Basic Molecular Genetic Methods for Identifying Dementia Causing Genes

II.2.a. Mapping and cloning disease-causing genes

Beginning in 1985, the author pioneered the use of molecular genetic strategies to dissect the causes and mechanisms of AD and other related dementias[70,71]. Multiple strategies have been developed to map and identify/clone genes causing susceptibility to dementia.

One commonly-used strategy has been to investigate datasets with families multiply affected with dementia using genetic linkage and/or family-based association methods. Genetic linkage studies, using maximum likelihood (LOD score) methods (Figure 4, left panel) to assess co-segregation of dementia with anonymous polymorphic chromosomal markers (e.g. RFLPs, microsatellites, idels, etc.), have been highly successful when used in rare families with clear-cut monogenic Mendelian autosomal dominant inheritance of dementia. This genetic linkage approach has permitted the chromo-

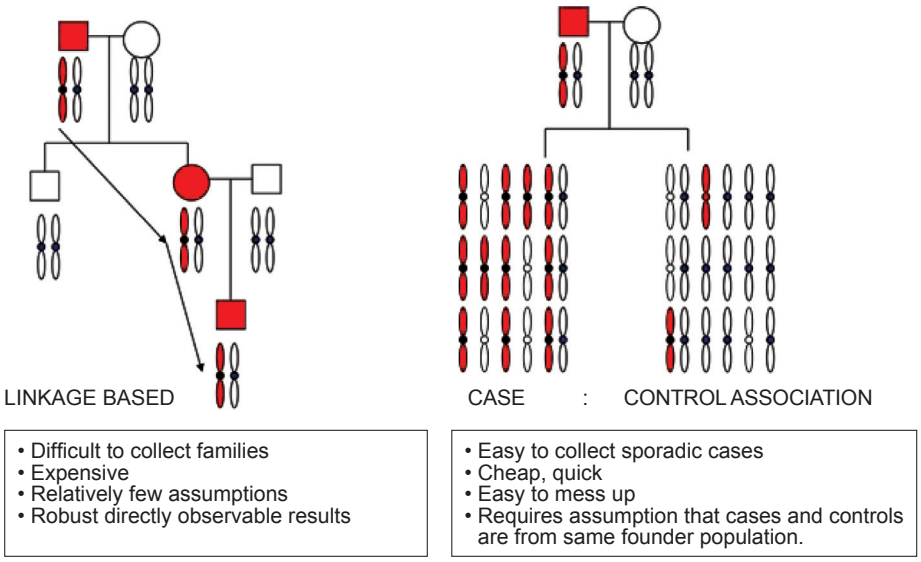


Figure 4: Genetic linkage studies, and to some extent family-based association studies rely on cosegregation of the trait and chromosomal markers to assign the location of a disease gene. In contrast, association-based methods (e.g. GWAS studies) look for differences in the frequency of a given allele in affected versus unaffected cohorts. This works best when the population is drawn from a homogeneous founder population so that all cases have the same genetic defect in the population. For admixed populations, where there are multiple different genetic variants causing the same disease, the power to detect association becomes less.

somal mapping and then the subsequent positional cloning of multiple genes causing AD and FTLD - including the APP and PS1 genes (see below).

Family-based association studies, such as FBAT, which are better suited to the analysis of families multiply affected by dementia, but where the inheritance pattern is less clear, have also been used. These studies led to the suggestion that, in AD for instance, there may be additional susceptibility loci in the pericentromeric region of chromosome 12 (Alzheimer Type 5)[72], the long arm and pericentromeric region of chromosome 10q (Alzheimer Type 6)[73], chromosome 20p[74], 18q[75], 15q22[76], and 9p[77]. However, replications were less robust and the identities of the putative AD genes at those locations (if they really even exist) have not been defined for any of these loci.

A third strategy to identify dementia-causing genes has been to use cohorts of sporadic cases and age/sex matched controls in a case:control

association design (Figure 4, right panel). The two cohorts (cases versus controls) can be genotyped with genetic markers. These markers have recently typically been single nucleotide polymorphisms (SNPs) and/or insertion-deletion polymorphisms (indels). These SNPs or indels can be either within individual genes of interest (i.e. a candidate gene approach) or can be incorporated into arrays of 0.5 – 1.5 million SNPs randomly distributed across the genome. This latter approach - a Genome Wide Association Study (GWAS) is currently highly favoured as a tool for dissecting complex disorders like AD. It allows a robust, unbiased, hypothesis free survey for susceptibility genes across the entire genome. In doing so, it allows analysis of genes that would never be considered as candidates. Thus it allows consideration of genes whose function was unknown or whose function was in pathways that would not have suspected to be important in the disease biology. As described below, this approach recently indicted several genes involved in inflammation, and in doing so reversed previous dogma about the relative importance of inflammation in these diseases. Previous ideas had suggested that inflammation was simply a late consequence of the disease. The discovery that inflammation pathway genes were in fact associated with susceptibility to the disease unequivocally revealed that inflammation was in fact a major and early component of the disease process. However, like all techniques GWAS approaches do have a number of important limitations including, most importantly, the fact that GWAS studies cannot detect genes in which there are multiple rare variants that occur on different SNP marker haplotypes.

The fourth approach, and one that is being increasingly pursued by the author's lab and many other labs in the field, is the use of "next generation" high-throughput DNA sequencing. This approach is well-suited to the discovery of rare variants that can frequently occur on different SNP haplotypes, and are thus not detected in standard GWAS studies. This high-throughput, deep sequencing technology can be focussed just on the coding sequences (Whole Exome Sequencing), or on the entire genome (Whole Genome Sequencing). The author's group together with colleagues[78] has recently used this approach to identify disease-causing mutations in the TREM2 gene in affected members of several late onset AD families.

II.2.b. Clarifying aetiological heterogeneity and gene interactions within pathways.

In addition to mapping disease-causing genes, molecular genetic approaches can be used to determine whether a disease is a single aetological entity or a constellation of several distinct diseases with a similar phenotype. Where the disease has several distinct causative genes, genetics can also answer the question as to whether those genes all interact in the same metabolic or signaling pathway, or whether they function in different pathways.

Use of both of these strategies has been massively informative in understanding the biology of the dementia.

A major discovery that arose from genetic studies by the author and colleagues related to the issue of heterogeneity. Until 1991, opinion was split as to whether AD was a single homogeneous disorder. One side of this argument believed that AD was a single disorder characterised by dementia with amyloid plaques and neurofibrillary tangles, and included the previous diagnostic categories of presenile dementia and Senile Dementia of the Alzheimer Type. There were other experts, however, who believed that AD was actually a series of causally and aetiologically distinct disorders that shared a common phenotype. This question was conclusively resolved by genetic studies performed by the author and colleagues[79]. Using genetic linkage methods, the author and colleagues conclusively demonstrated that a subset of AD cases arose from defects in a gene on chromosome 21, while other cases arose from genetic defects on other chromosomes and/or from non-genetic aetiologies.

While this result now sounds trivial, it had an ***immediate and profound*** effect on both basic research and clinical research on this disorder. The clarification that the disorder was aetiologically heterogeneous allowed focused attempts to identify the gene on chromosome 21 (which turned out to be APP), and to map and clone the remaining genes on other chromosomes. The clarification that the disorder was aetiologically heterogeneous also then required that clinical studies (including clinical trials) interpret their data by bearing in mind the possibility that the clinical cohorts under study were not homogeneous and identical. It is now entirely routine to conduct post-hoc, and at times even *a priori* analyses of clinical trial data by taking into

consideration the genotype at APOE (i.e. classifying participants into APOE ε4-positive and APOE ε4-negative subgroups).

The key role of genetics is deciphering which genes act in the same pathway, and identifying that pathway, is described in greater detail in the Section III below on the genetics of Alzheimer's disease. In AD, several genes have been found to map into metabolic pathways that affect APP processing and Aβ production, or that affect the downstream pathways activated by the accumulation of Aβ aggregates (e.g. activation, by Aβ aggregates, of complement-mediated innate immune and microglial-mediated neuroinflammation pathways (see Figures 8 and 23 below).

III
Discoveries Arising From Basic Science Studies of Specific Neurodegenerative Dementias

III.1. Alzheimer's disease

Alzheimer's disease (AD) is the most common degenerative cause of dementia. It typically begins in mid-late adult life with the progressive onset of memory loss that is accompanied by progressive impairments in at least one other cognitive function (e.g. judgement, apraxia of previously learned skills, etc) (Figure 5). There are usually no focal neurological deficits (e.g. paresis,

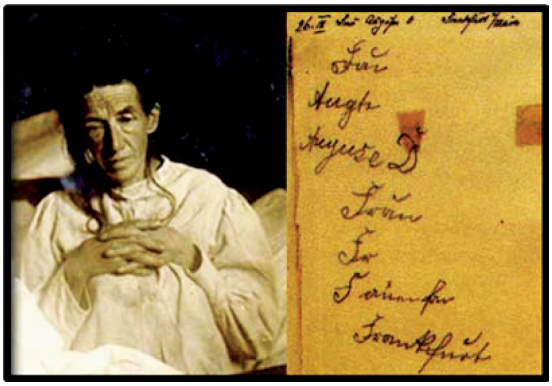


Figure 5: Auguste D., Alzheimer's first patient in 1901. Script demonstrates cognitive impairment with perseveration.

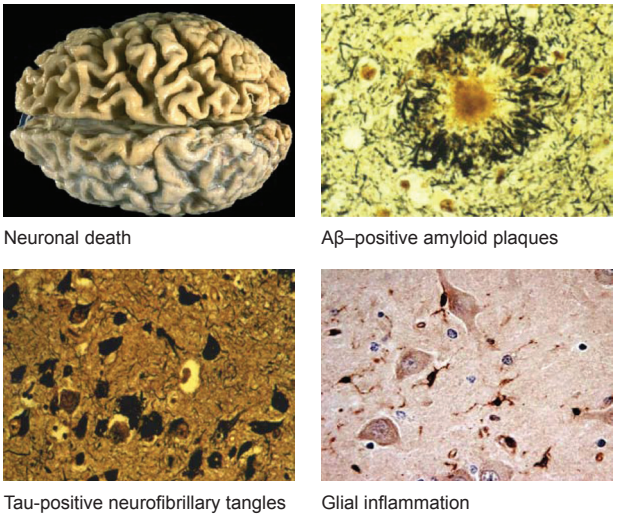


Figure 6: The principle pathological features of Alzheimer Disease.

blindness). These cognitive defects are progressive and lead inexorably to a bedridden state, and subsequently to the demise of the patient usually through inter-current illnesses. These clinical features are accompanied by a set of relatively stereotyped neuropathological changes including widespread loss of neurons from the forebrain, particularly in the frontal, temporal, and parietal association cortices (Figure 6). This loss of neurons can be documented during life by structural Magnetic Resonance Imaging (MRI) and Computed Axial Tomography (CAT) scanning. This gross neuropathological change is accompanied by at least three characteristic neuropathological changes: 1) in the extracellular space of the cortex, the presence of spherical deposits of amyloid β-peptide (Aβ) that are frequently surrounded by glial and neuritic reaction (diffuse and compact amyloid plaques); 2) the presence inside cortical neurons of paired helical filament inclusions composed of the microtubule associated protein tau (neurofibrillary tangles - NFTs); and 3) evidence of activation of cerebral inflammation characterized by activated microglial and astrocytic cells.

Recently, although the diagnostic clinical and pathological features of AD described by Alois Alzheimer[10] are still broadly correct, the diagnostic criteria for AD have been updated[80]. This update was done to allow the inclusion of information from biomarkers that are thought to reflect the mechanistic processes underlying this disease (see below for details on these processes). These major biomarker categories include: 1) changes in the amount of tau protein in the CSF (total tau and phosphorylated tau); 2) decreased metabolic function in the temporal and parietal cortex as measured by ^{18}fluorodeoxyglucose (FDG) uptake in Positron Emission Tomographic (PET) imaging in the temporoparietal cortex; and 3) disproportionate atrophy in the medial basal and lateral temporal lobe and medial parietal cortex.

These new criteria have been formulated for the clinical diagnosis of: possible / probable dementia due to Alzheimer's disease[81]; for Mild Cognitive Impairment (MCI) due to Alzheimer's disease[82], and preclinical Alzheimer's disease[83]. There are also improved criteria for neuropathological confirmation of AD[84].

The *current* management of cases with suspected, possible or probable Alzheimer's disease is mainly directed at the symptoms of AD. This symptomatic focus is because there are currently no approved disease-modifying therapies. This symptomatic management is directed at improving some as-

pects of cognitive function and attention using acetylcholinesterase inhibitor compounds (e.g. donepezil (Aricept®), rivastigmine (Exelon®)). These drugs block degradation of acetylcholine released from cholinergic neurons in the nucleaus basalis of Meynert, which are known to degenerate in AD[85-87]. Cholinesterase inhibitors are often supplemented with memantine (Exiba®), a low affinity noncompetitive antagonist at glutaminergic NMDA receptors. Symptomatic management of the other affective and behavioural symptoms of AD (apathy, depression, anxiety, wandering, sleep/wake diurnal disturbances, outbursts) are typically achieved through a combination of non-pharmacological and pharmacological interventions.

Unfortunately, none of these pharmacological and non-pharmacological behavioural interventions[88-91] work well. Some, such as neuroleptics, are even significantly risky[88-90]. As a result, the long-term prognosis for patients diagnosed with AD is universally poor, with death occurring typically within 10-15 years after diagnosis. As far as can be determined, there are no cases of Alzheimer's disease that have undergone spontaneous remissions. The quality of life for sufferers is typically grim in the final stages of the disease. Moreover, the disease exerts significant an emotional (and very often financial) toll on family and non-paid caregivers as well as professional caregivers[92,93].

In light of its frequency, it's growing significance as a future public health problem, and it's currently intractability to even symptomatic management, there is a growing urgency to find effective disease-modifying therapies, and better yet, treatments that will actually cure or prevent the disease.

Clearly, the rational discovery of effective disease-modifying/curative or preventative therapies for AD must be built upon a detailed understanding of the causes and molecular mechanism of the disease. The following paragraphs describe the current state of knowledge about the aetiology and molecular pathogenesis of AD that has emerged from the last two decades of concerted work in molecular genetics, molecular biology, cell biology and animal modelling of AD by multiple groups, including work from the author's group.

III.1.a Genetic Basis of Alzheimer Disease

Epidemiology studies undertaken on probands with Alzheimer Disease (AD) and their families reveal that the overall lifetime risk for AD in first-degree

relatives of AD probands is about 38% by age 85 years[94]. These studies also demonstrate that genetic factors account for about 40% of the population risk for AD. In about 5-10% of cases, AD appears to be inherited as a simple monogenic trait with an autosomal dominant pattern of transmission. However, in the majority of cases these genetic factors are manifest by the presence of multiple family members affected with AD, but without a clear-cut Mendelian pattern of inheritance ("multiplex pedigrees"). Several possible modes of transmission could account for these "multiplex" familial cases. These include transmission due to: 1) an incompletely-penetrant, single, autosomal dominant gene defect; 2) a complex trait arising from the synergistic effects of multiple genes, each with small effect sizes (termed "oligogenic" or "multigenic" inheritance); or 3) complex interactions between genetic and environmental factors.

The entire set of molecular genetic methods described in Section II have been used to dissect the genetic defects causing AD in: a) rare families with simple monogenic autosomal dominantly inherited AD; b) families with oligogenic multiplex clusters of AD; and c) cohorts of apparently "sporadic" cases and normal controls. Over the last 20 years, this broad approach to the genetics of AD has led to the discovery of at least 15 different genes associated with varying degrees of risk for AD. It is suspected that additional, but as yet unidentified, AD susceptibility genes must exist because only about 50% of the overall risk attributable to genetic factors is accounted for by the ~15 genes that are known to date. Nevertheless, as will be seen below, it is increasingly apparent that the currently-known AD-susceptibility genes interact in a series of interrelated pathways governing: a) APP processing and intracellular trafficking; b) the production and clearance of Aβ fragment of APP; and c) the downstream consequences of the accumulation of Aβ. The latter include: activation of complement-mediated innate immunity and glial inflammation; defective cholesterol/lipid metabolism; defective proteostasis of other proteins such as tau, α-synuclein, or TDP-43 (neuropathological deposits of which are also commonly seen in AD brain tissue[38-40,95-100]).

III.1.a.i. Amyloid Precursor Protein

The first gene to be associated with inherited susceptibility to AD was the amyloid precursor protein gene (APP). The APP gene was cloned and

mapped to chromosome 21 by several groups[16-18], including the author's group[15,19]. We and others showed that the APP gene produces an alternatively spliced transcript which, in its longest isoform, encodes a single transmembrane spanning polypeptide of 770 amino acids (Figure 7)[16].

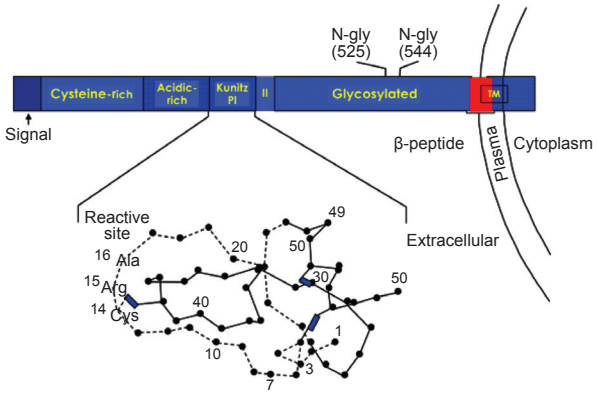


Figure 7: Cartoon of the topology of the APP protein domains. The Aβ domain is depicted by the red box spanning the C-terminus of the extracellular domain and half of the TM donmain, and is generated by cleavage of the APP holoprotein.

Following trafficking to the cell surface, the APP precursor protein is either recycled into the secretory pathway and then back to the cell surface via endosomes, or it undergoes a series of endoproteolytic cleavages (Figure 8). The majority of APP is either: 1) recycled back and forth between the cell surface and the retromer; or 2) it undergoes cleavage mediated by the membrane-associated ADAM10 and ADAM17 disintegrin metalloproteases. These membrane-bound enzymes cleave APP_{695} in the middle of the Aβ peptide domain. This cleavage is termed α-secretase and precludes the formation of Aβ.

Lesser amounts of APP (~5 - 10%) are processed by a minor cleavage pathway that occurs principally after internalization from the cell surface in the late endosome compartment, and involves sequential cleavages by β- and γ-secretases, which then generate a 40-42 amino acid peptide termed the Aβ peptide (Figure 8). The first of these cleavage events occurs at the beginning of the Aβ domain, and is mediated by β-secretase (BACE 1), a Type 1 transmembrane glycosylated aspartyl protease[101]. This cleavage generates a solu-

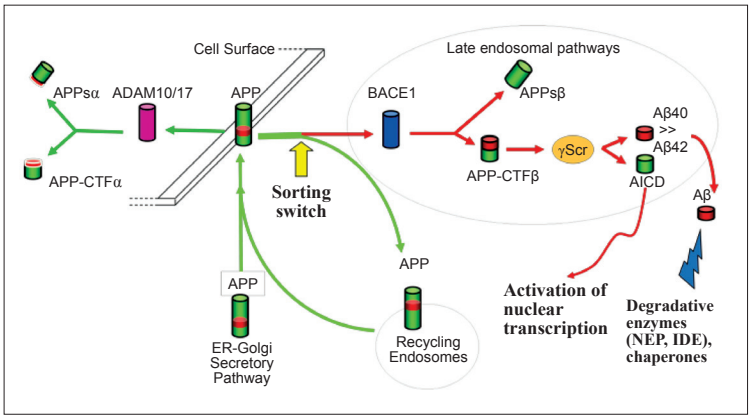


Figure 8: Proteolytic processing pathways for APP. Only the BACE + γ-secretase pathway generates Aβ. AICD is translocated to the nucleus.

ble N-terminal fragment ($APP_{s\beta}$), and a membrane-bound stub (APP-C100). The second set of cleavages, termed γ-, ζ- and ε-secretase cleavages, occur at several sites between residues +38 and +59 of the membrane-bound APP-C100 stubs. The γ-, ζ- and ε-cleavages are performed by the presenilin complex (see Section III.1.aiii below). The N-terminal product of the γ/ε-secretase cleavage of APP-C100 is Aβ, while the C-terminal product is a labile fragment termed Amyloid Intra-Cellular Domain (AICD). The AICD might be translocated to the nucleus where it acts as a signal transduction molecule[102]. The γ/ε-secretase cleavage actually generates a mixture of Aβ peptides containing 38, 40, 42, or 43 amino acids. Aβ peptides ending at residue 42 or 43 (long tailed Aβ) are thought to be more fibrillogenic and more neurotoxic than Aβ ending at residue 40, which is the predominant isoform produced during normal metabolism of APP. Little is currently known about the physiological role (if any) of Aβ. Aβ is removed by several pathways, including degradation by a variety of peptidases such as neprilysin[103], plasmin, and insulin degrading enzymes (IDE)[104] (Figure 8).

The function of APP is currently unknown. Knockout of the murine APP gene leads only to minor weight loss, decreased locomotor activity, abnormal forelimb motor activity, and non-specific degrees of reactive gliosis in the cortex. However, double knockouts of APP and one or more of its homo-

logues (termed amyloid precursor-like proteins – APLPs) cause embryonic lethality, suggesting that APP and the APLPs have redundant but essential activities[105]. *In vitro* studies in cultured cells suggest that the N-terminal secreted fragment of APP (APPs) can function as an autocrine factor, stimulating cell proliferation and cell adhesion. Other studies have implied a role for APP in: 1) signal transduction by association of APP with heterotrimeric GTP-binding proteins; 2) a receptor for kinesin-1 during the fast axoplasmic transport of vesicles containing BACE and presenilins, where the cleavage of APP serves to stop the kinesin-mediated trafficking of the transport vesicle; and/or 3) a signal transduction molecule in a manner similar to Notch signalling. The C-Terminal fragment of Notch that is equivalent to AICD is the Notch Intra-Cellular Domain (NICD). Following binding of Delta to Notch at the cell surface (during dorsal axis development in embryogenesis, The presenilin complex cleaves the Notch protein within the membrane, and releases NICD into the cytoplasm. NICD (and presumably also AICD) is then translocated to the nucleus, where it transcriptionally activates multiple downstream genes involved in Notch signalling (Figure 8).

Several observations made by the author and by many other researchers beginning with George Glenner[11], led to the hypothesis that mutations in the APP gene might cause AD. First, a fragment of APP (Aβ) was part of the pathology of AD[11]. Second, individuals with trisomy 21 develop AD-like changes in their brains[106]. Third, the cloning and mapping of the APP gene to chromosome 21 nearby markers that co-segregated with AD in families by the author[15,19] and colleagues[20] implied that mutations in APP might cause AD. Fourth, sequencing of exons 16 and 17 in the APP gene of patients with Hereditary Cerebral Haemorrhage with Amyloidosis (HCHWA) led to the discovery of the first pathogenic mutations in APP[107]. Direct sequencing of the APP gene by several groups[108-111], including the author's group[112], subsequently uncovered at least 25 different AD-associated missense mutations in the APP gene (most in exons 16 and 17) in families with early-onset AD (http://molgen-www.uia.ac.be/ADMutations).

All known AD-associated mutations in APP either alter APP processing and Aβ production, or alter the propensity of the resulting Aβ peptide to aggregate into β-sheet amyloid fibrils. Some of the missense mutations in the APP gene result in the relative (APP_{717}) or absolute ($APP_{670/671}$) over-

production of full length Aβ species ending at residue 42. Other mutations cause the over-production of N-terminally truncated species of Aβ ending at residue 42 (APP_{715}); or the production of Aβ species that have increased propensity to assemble into neurotoxic fibrils (APP_{692}, APP_{693}).

Multiple molecular mechanisms have been proposed to explain the neurotoxic effects of Aβ (and especially of small soluble aggregates variously called oligomers, amyloid-derived-diffusible-ligands or ADDLs, and protofibrils) (See Section II). These include both direct effects (e.g. inducing apoptosis by effects on cell membranes) and by indirect effects (e.g. potentiating effects of excitatory amino acids on NMDA receptors, oxidative stress, and increases in intracellular calcium and free radicals). However, Aβ may not be the only cytotoxic product of β- and γ-secretase cleavage because the cytoplasmic C-terminal stub (C31-APP) is also toxic when over-expressed. This series of discoveries has had huge practical significance. They have led to the invention of both novel biomarkers (e.g. CSF Aβ assays; position emission tomography (PET) imaging agents such as PIB); and also candidate therapies (e.g. Aβ vaccines; Aβ aggregation inhibitors) (see Section IV below).

III.1.a.ii. Apolipoprotein E

The association of APOE with inherited susceptibility to typical "sporadic" late onset AD was uncovered by the concurrence of four lines of investigation. First, genetic linkage studies in pedigrees with predominantly late-onset, familially aggregated AD provided suggestive evidence (z = +2.5) for an AD susceptibility locus on chromosome 19q12-q13 near the APOE gene. Second, analysis of proteins from the CSF that were capable of binding the Aβ peptide revealed that one of the proteins was apolipoprotein E (APOE). Third, APOE is a biochemical component of the senile plaque of AD. Finally, genetic association studies by the author and Allen Roses revealed that one allelic variant of APOE (ε4) was associated with a large (13x) increase in risk for "sporadic" late onset AD[113].

The APOE gene in humans contains three common polymorphisms - ε2 (cysteines at codon 112 and codon 158); ε3 (cysteines at codon 112); and ε4 (arginine at codon 112). Analysis of these polymorphisms in normal control

populations and in patients with AD by Allen Roses' group in collaboration with the author's group[113], led to the discovery that there is an increase in the frequency of the ε4 allele in patients with AD (allele frequency in AD is approximately 40%, compared to 15% in normals), and that there is a smaller reduction in the frequency of the ε2 allele (from 10% to about 2% in AD). More significantly, there is a dose-dependent relationship between the number of copies of ε4 and the age-of-onset of AD such that each copy of ε4 reduced age-of-onset by 10 years. Thus, ε4/ε4 subjects have an earlier onset than do heterozygous ε4 subjects. Subjects with the apparently protective ε2 allele, on the other hand, have a later onset. The association between ε4 and AD has been robustly confirmed in numerous studies and in several different ethnic groups. The association is weaker with advanced age of onset.

Although the association between APOE ε4 and AD is robust, it is not specific. Observations in patients with head injury[114], spontaneous intracerebral haemorrhage[115], and in patients undergoing elective cardiac bypass surgery[116], all suggest a poorer outcome for patients with the ε4 allele. There is a confirmed association between the ε4 allele and the Lewy body variant of AD[117].

The mechanism by which the ε4 allele is associated with an earlier onset of AD, and by which the ε2 allele is associated with a later onset is unclear. The most obvious hypothesis is that APOE might influence the production, distribution, or clearance of the Aβ peptide. This hypothesis is supported by observations by the author and[118] others[119] that the genotype at APOE modulates age-of-onset in subjects carrying the APP Val717Ile mutation (but not the APP_{692} mutation), suggesting a direct biochemical interaction between APOE and APP (or its metabolic products) (see Figure 30 and Section IV, 2b below). Second, subjects with one or more APOE ε4 alleles have a higher Aβ peptide plaque burden than do subjects with no ε4 alleles[120]. *In vitro* studies suggest that delipidated APOE ε4 binds Aβ more avidly than APOE ε3. There is also evidence that both APOE and Aβ may be cleared through the lipoprotein-related (LRP) receptor and/or via the LDL-receptor and that APOE ε4 and the Aβ peptide may compete for clearance through the LRP and LDL-R receptors. Finally, transgenic mice expressing the APP_{V717F} mutation (PDAPP mice) develop profound cerebral Aβ deposition when bred on an $APOE^{+/+}$ background, but have very little Aβ deposition on an $APOE^{-/-}$ background.

However, the fact that APOE ε4 is not specifically associated with AD raises the possibility that it acts by some other non-specific "off pathway" mechanism. Along this line of thinking, an alternate hypothesis relates to changes in cholesterol metabolism in AD and their effects on Aβ metabolism. Both epidemiological and direct experimental evidence in cell culture models suggests that cholesterol metabolism and APP metabolism are functionally intertwined. Specifically, reduction in cellular cholesterol availability results in significant changes in APP trafficking and processing, with the resultant reduction in Aβ formation[121]. In addition, epidemiological studies on patients who have taken statins for hypercholesterolemia appear to have a reduced incidence of Alzheimer Disease (although statins have no significant effect of the disease in patients with clinically diagnosed AD)[122].

Finally, there is a good correlation between the degree of clinical dementia and the decrease in synaptic density in AD, and it has been suggested that APOE may be involved in synaptic plasticity during regeneration and repair, and that the ε4 allele is less efficient in this role. This is in accord with clinical epidemiological data suggesting that the presence of APOE ε4 is associated with: 1) lower pre-morbid IQ scores in young adults; and 2) with a poorer outcome after a variety of unrelated CNS injuries including head injury, stroke, and coronary artery bypass grafting. It has therefore been suggested that the association between APOE ε4 and AD may not determine whether AD occurs, but rather, the clinico-pathologic response to other causative factors by modulating a variety of effects including Aβ processing and regeneration-repair etc[123]. Indeed, these putative effects of APOE on several different mechanisms need not be mutually exclusive.

Regardless of the underlying mechanisms, the discovery of the association of AD with APOE ε4 by the same author and colleagues[113] has had a *profound* effect on both the the understanding of late onset "sporadic" AD, and increasingly on the management of patients with typical late-onset sporadic AD. Thus, APOE genotype can be used as an adjunctive clinical test. APOE Is routinely used to partition clinical trial cohorts into more homogenous (APOE- vs APOE+) subgroups. And APOE has recently been used as a therapeutic target in preclinical drug trials. Specifically, bexarotene has been shown to upregulate APOE levels which then caused, with a few days, a dramatic clearance of A plaques and significant improvement in cognitive function in APP transgenic mice[124].

III.1.a.iii. Presenilin 1

Following the sentinel discovery by the author that AD was genetically heterogeneous[79], and that only a small proportion of families with autosomal dominant AD had mutations in the APP gene[125], the author's group set out to identify the chromosomal location of other genes causing autosomal dominant familial AD, and then identify the causative genes. Genetic linkage studies by the author's group[126] and others located a third Alzheimer susceptibility locus (AD3) to a region of approximately 10 centiMorgans on the long arm of Chromosome 14. The author's group then cloned the actual disease gene by using a positional cloning strategy[127]. The underlying gene, which the author named Presenilin 1 turned out to be a previously unknown gene, the characterization of which would come over the next decade, have huge implications for both our understanding of AD and for basic biology.

To date, more than 155 different mutations have been discovered in the PS1 gene by the author's group and by others (http://molgen-www.uia.ac.be/ADMutations). The majority of these mutations are missense mutations giving rise to the substitution of a single amino acid. A few in-frame splicing, deletion or insertion defects have also been identified. However, nonsense mutations resulting in truncated proteins have not been found in AD-affected

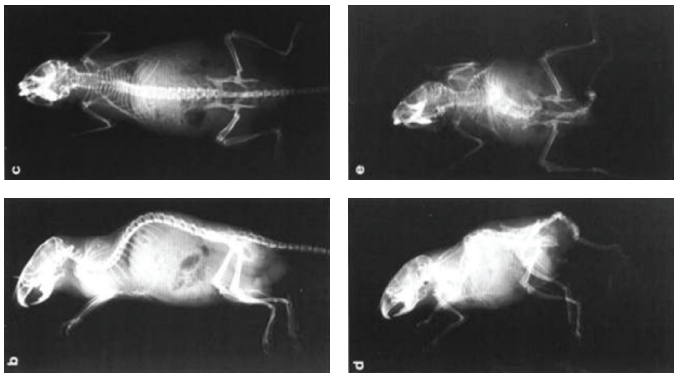


Figure 9: Loss of the presenilin complex causes defective cleavage of Notch required for Notch-Delta signaling during dorsal axis development in embryogenesis. As a result PS1 null mice have a severe defect in the spinal cord, like Notch knockout mice.

subjects. We[8] and other groups[128] have shown that all of these clinical PS1 mutations associated alter γ-secretase cleavage of APP, and lead to a *relative* increase in production of toxic long-tailed Aβ peptides ending at residue 42/43[8]. In strong contrast to the effects of clinical AD-associated mutations, artificial null mutations in the presenilins result in severe developmental defects (Figure 9)[129]. This disparity indicated early on that the effect of clinical mutations was to induce an aberrant function, not complete loss of function. However, both of these effects arise because the presenilin proteins are a key component of a tetrameric protein complex (see next paragraph) that is involved in an unusual form of proteolysis of several type I transmembrane proteins including APP (defining PS1's relationship to AD) and Notch (defining PS1's relationship to developmental defects). The molecular and structural biology underlying these events is described in the paragraphs below.

III.1.a.iii.a Biochemistry and Molecular Biology of Presenilin 1

The author's group showed that the presenilin 1 (PS1) gene is highly conserved in evolution, being present in *C. elegans* and *D. melanogaster*. PS1 encodes a polytopic (multi-spanning) integral membrane protein with eight transmembrane domains, and with a large, hydrophilic, acidically charged loop domain between the putative sixth and seventh transmembrane domains (Figure 10). The PS1 protein is approximately 50 kDa in size and is predominantly located within intracellular membranes in the endoplasmic reticulum, the perinuclear envelope, the Golgi apparatus and at the cell surface as well as in some as yet uncharacterized intracytoplasmic vesicles (Figure 11). Only very small amounts of the PS1 holoprotein exist within the cell at any given time. These small amounts of the PS1 holoprotein may form slow leak calcium channels, using a central solute accessible channel in the protein structure (see Figure 14, Section III.1.a.iii.c below)[130]. However, the majority of the holoprotein undergoes a self-catalysed endoproteolytic cleavage near residue 290 within the TM6-TM7 loop domain to generate N- and C-terminal fragments (NTF and CTF) (Figure 10). The PS1-NTF and PS1-CTF are the principle biologically active form of the protein. They remain tightly associated with each other in a high molecular weight mul-

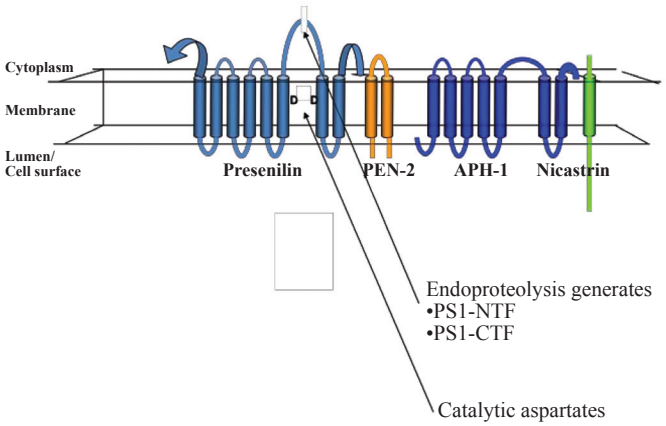


Figure 10: The presenilin proteins form tetrameric high molecular weight complexes with presenilin enhancer 2 (PEN-2), anterior pharynx 1 (APH-1) and nicastrin. The loop domain between the two TM domains contain the catalytic aspartates (TM6 and TM7) undergoes endoproteolysis during activation of the complex.

timeric protein complex that acts as the γ-secretase enzyme (see Figure 14, Section III.1.a.iii.c below).

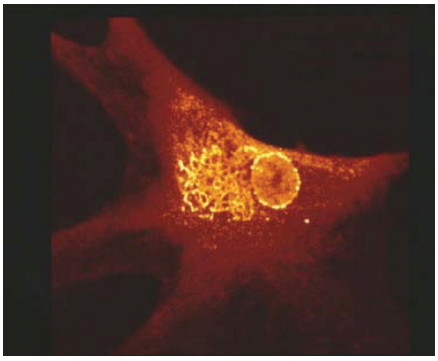


Figure 11: Confocal micrograph of endogenous PS1 expression in a neuron. The immunoreactivity is predominantly in intracellular membranes.

The author's group was the first to show that the presenilins are components of a high molecular weight complex, and it is this complex that contains the active form of the presenilin NTF and CTF[131]. We showed that

the complex contains three other core proteins (Figure 10), plus two or three optional regulatory proteins (shown in Figure 13). The first of the three other core proteins to be discovered was nicastrin (a ~110kDa Type 1 transmembrane glycoprotein), which we were the first to identify using a novel mass spectrometry approach[131]. We were also the first to show that nicastrin may act as the substrate binding molecule[132]. The two other component proteins are: APH-1 (a polytopic transmembrane protein that may act as the initial assembly molecule for the complex[133]); and PEN2 (a short hydrophobic protein with two transmembrane domains, but with unknown function). We have shown that the core proteins actually cluster into two hemi-complexes, which remain together and form the fully functional holo-complex. One of the hemi-complexes contains PS-NTF and PEN-2. The other hemi-complex contains PS1-CTF, APH-1 and Nicastrin. As will be described below, this structural arrangement is likely to be functionally significant (see Section III.1.aiii.b below). The optional regulatory proteins include TMP21 discovered by the author's group[134], and gSAP, discovered by the Nobel laureate Paul Greengard[135]. We and Greengard have shown that these proteins selectively modulate specific proteolytic cleavage site activities (γ versus ε) being conducted by the core component proteins (Figure 13).

III.1.a.iii.b Cell Biology of Presenilin 1 mediated intramembranous proteolysis

We[8] and others[128,136] have shown that the presenilins play a catalytic role in the proteolytic processing ("Regulated Intramembranous Proteolysis") of several Type 1 transmembrane proteins including APP, p75, LRP, ErbB4, and Notch. Regulated Intramembranous Proteolysis is a term coined by the Nobel Laureates Michael Brown and Joseph Goldstein for a previously unrecognized form of protease activity performed by membrane-bound proteases that cleave their substrates with the substrates transmembrane (TM) domains (Figure 12)[7]. The catalytic residues (typically serine, aspartate) of these unusual enzyme are also located on one or more TM of these enzymes.

The presenilin-mediated cleavage of its substrates occurs at three sites within the substrate's TM domains (Figure 13). The initial cleavage event likely occurs close to the inner (cytoplasmic) leaflet other membrane, and

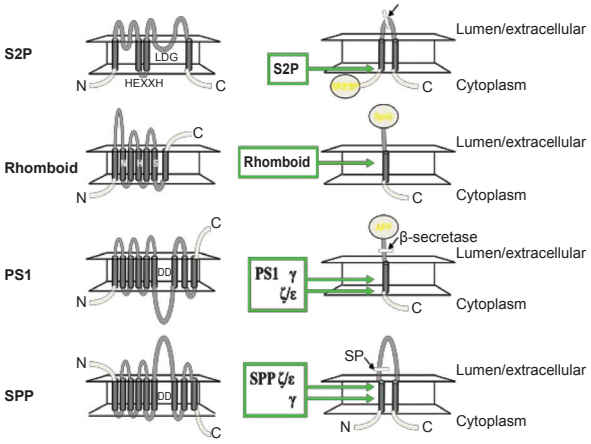


Figure 12: The presenilin proteins perform a novel type of proteolysis in which the substrate proteins are cleaved within their TM domains. Unexpectedly, this hydrophilic cleavage reaction occurs within the hydrophobic compartment of the membrane itself. Only four enzymes are known to do thjs: Site 2-proteases (S2P), Rhomboids, presenilin complexes (PS1) and signal peptide peptidases (SPP). SPPs and PS1/PS2 are homologous members of the same family, but have opposite topology and preferences for Type 2 (SPP) versus Type 1 (PS1/PS2) single spanning membrane proteins as substrates.

is termed ε-site cleavage, which generates the C-terminal cytoplasmic fragment (Amyloid Intra Cellular Domain or Notch Intra Cellular Domain). The next cleavage event is the ζ-site cleavage which generates a small labile fragment. The final cleavage event is the β-site cleavage which generates the N-terminal (Aβ) peptide fragment and a small labile fragment.

III.1.a.iii.c Structural Biology of Presenilin 1 mediated intramembranous proteolysis

The structural biology of PS1 cleavage is an intriguing biological and biophysical enigma that is slowly being unfolded through structural biological work by the author and a few other groups. Despite the extreme clinical and biological importance of presenilin-mediated proteolysis, little is known about the structural mechanics of how substrates are selected by the presenilin complex. It is also unclear how substrates gain access to the active catalytic site of the complex, or how free water molecules are able to access the catalytic site. This catalytic site is likely buried within a hydrophobic pocket

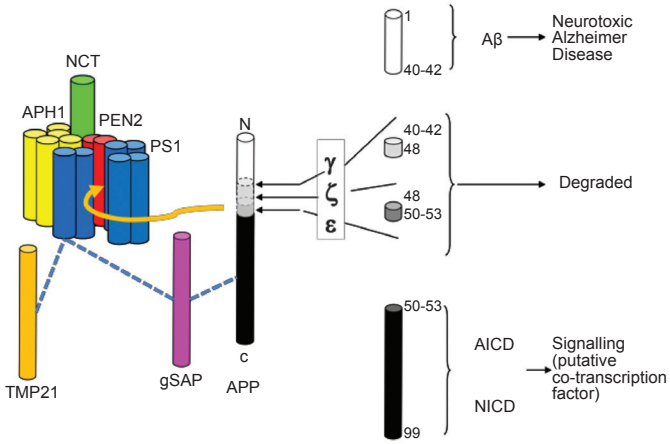


Figure 13: The presenilin complex, composed of the N-NC-terminal fragments of PS1 *(blue)*, PEN-2 *(red)*, APH1 *(yellow)* and nicastrin *(green)* likely acquires the TM domains of substrates via lateral motion in the plane of the membrane *(orange arrow)*. Substrates bind to the PS1-NTF and-CTF at the Initial Substrate Binding Site, and are then translocated into the active site composed of the catalytic aspartate residues on TM6 on PS1-NTF, and TM7 on PS1 and CTF. A series of cleavages are performed at three different sites (ε, ζ and β), thereby generating four different fragments. These cleavages are under independent control, and can be modulated by TMP21 *(orange)* or gSAP *(purple)*. The stable products of this cleavage are the N-terminal Aβ peptide *(white)* and the C-terminal cytoplasmic soluble stub (AICD/NICD - *black*). The Aβ peptide gives rise to AD. The AICD/NICD fragments are translocated to the nucleus, where they may activate transcription of downstream signalling pathway genes.

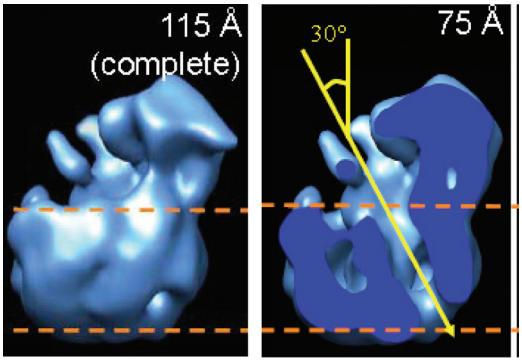


Figure 14: 15Å resolution 3D single particle electron density map of intact functional human PS1 complex generated by the author (submitted for publication). **Left: surface view** (115Å) showing the exposed head domain (containing the glycosylated ectodomain of nicastrin) and the membrane-embedded body (containing the transmembrane domains of the component proteins)(*dotted orange lines depict membrane boundaries*). **Right: cut-away view** through the centre of the complex, showing internal solute accessible channel (traversed by the *oblique yellow line*) which likely harbours the catalytic aspartates in the active site, provides a source of catalytic H2O and may act as an ion channel.

within the transmembrane domains of the complex. It has been presumed that the TM domains of substrates would gain access by lateral movement in the plane of the membrane between the TM domains of the PS1 complex proteins – i.e. via a putative "lateral gating" mechanism (Figures 13, 14).

Similarly, high throughput screening of chemical libraries has yielded numerous small molecule inhibitors and modulators to block the production of Aβ peptide as a potential treatment of Alzheimer's disease (AD)[137]. Some of these compounds resemble classical, transition state analogue, active site inhibitors of soluble aspartyl proteases. However, many inhibitors and modulators do not act at the active site[137]. Some of these non-transition state inhibitor/modulator compounds bind to the PS1-NTF at sites that are distinct from both the catalytic and the initial substrate docking sites[138-144]. The structural basis for the activity of these atypical inhibitor/modulator compounds also remains unknown.

To address these questions we have used single particle electron microscopy (EM) reconstruction at the level of 15Å resolution, intra-molecular Förster Resonance Energy Transfer (FRET) efficiency analysis and ligand binding assays to investigate the three dimensional structure of native PS1 complexes and of complexes with a non-transition state inhibitor (Compound E[140]) bound (Figure 14).

These studies (submitted) provide, for the first time, three novel observations about the structure and function of the presenilin complex. First, our work provides new details about the structure of the presenilin complex, revealing that it has a bilobed shape with a distinct head domain that contains the ectodomain of Nicastrin. There is a central solute accessible channel which opens on both the external and internal surfaces (Figure 14). Second, our work reveals, again for the first time, that some peptidomimetic compounds inhibit γ-secretase activity by binding to PS1-NTF and inducing allosteric conformational changes at the Initial Substrate Docking Site. These allosteric effects interfere with the binding and translocation of substrates to the active site. The importance of this observation is that a similar mechanism may be used by γ-secretase modulator (GSM) compounds - a class of clinically-highly-promising compounds that selectively inhibit γ-cleavage of APP without affecting ε-cleavage of Notch[144-146]. Third, our work suggests that the putative "lateral gate" mechanism likely works through a series of reciprocal allosteric interactions at the interface between the two hemi-com-

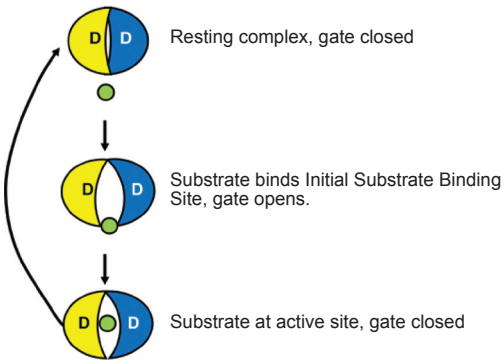


Figure 15: Putative "lateral gate" mechanism by which substrate binding to the Initial Substrate Binding Site, which is comprised of PS1 NTF hemi-complex *(yellow)* and the PS1-CTF hemi-complex *(blue)* results in allosteric opening of the translocation pathway towards the catalytic aspartate residues on each hemi-complex ("D"). Subsequent occupancy of internal sites in the translocation pathway results in reciprocal allosteric changes that close the substrate binding site behind the substrate, thereby protecting the active site residues and preventing further substrate entry. Cleavage of the substrate results in release of fragments and the return of the complex to the resting state with the gate closed.

plexes (see above) (Figure 15). Following binding of the substrate TM (at the interface of PS1 NTF and PS1-CTF), these reciprocal allosteric interactions likely set up a gating mechanisms that allows translocation of the substrate TM between the hemi-complexes towards the two catalytic aspartate residues - one located on TM6 of the PS1-NTF/PEN-2 hemi-complex; the other located on TM7 of the PS1/APH1/nicastrin hemi-complex (Figure 15). This reciprocally-operated gating mechanism would also account for the relatively slow processivity of γ-secretase cleavage[147].

Further enhancement of the resolution of these structural studies using cryo-EM of the whole complex, and using NMR and X-ray crystallography studies of the component proteins, will render further knowledge about the mechanics of this very important but very unusual enzyme. Such structural information will be essential for the improved design of novel modulators of γ-secretase activity. Such compounds, when tuned to selectively reduce Aβ42 without affecting Notch cleavage, will have utility in the treatment and prevention of disease in carriers of APP, PS1 and PS2 mutations (where the disease defect is at this step in Aβ production). These drugs may also have utility for other forms of AD. Conversely, compounds tuned to inhibit Notch signaling will have utility in the treatment of some leukemias.

III.1.a.iv. Presenilin 2

During the cloning of the presenilin 1 gene on chromosome 14, we identified for the first time, and then cloned a previously unknown, but homologous sequence (Presenilin 2-PS2) on chromosome 1[148]. We showed that PS2 encodes a polypeptide whose open reading frame contains 448 amino acids. This open reading frame sequence has substantial sequence similarity to PS1 (overall identity is ~60%), and a very similar structural organization. Despite this similarity, PS1 and PS2 have distinct although partially overlapping functions. For instance, we have shown that PS2 is not able to functionally replace either the APP or Notch processing defects in PS1 Knockout animals[149-151]. However, PS2 mutations, like PS1 mutations, increase the secretion of long-tailed Aβ42/43 peptides[152]. These observations suggest that APP and Notch are probably not the preferred substrates of PS2 complexes. The preferred substrates for PS have not yet been identified.

Mutational analysis of the PS2 gene led by the author's group[153] and subsequently by others have uncovered a small number of missense mutations (~10) in the presenilin 2 gene in families segregating autosomal dominant AD (http://molgen-www.uia.ac.be/ADMutations). The phenotype associated with PS2 mutations is much more variable[154]. Thus, the vast majority of heterozygous carriers of missense mutations in the APP and PS1 genes develop the illness between the ages of 35 and 55 years for PS1 mutations, and between 45 and 65 years for APP mutations. In contrast, the range of age-of-onset in heterozygous carriers of PS2 mutations is between 40 and 85 years, and there is at least one instance of apparent non-penetrance in an asymptomatic octogenarian transmitting the disease to affected offspring[155]. The reason for this may be that APP is not the preferred substrate for PS2 and so different mutations may have different effects on Aβ42 production.

III.1.a.v. SORL1 and other VPS10 sorting protein genes

The growing evidence that trafficking of APP from the cell surface was required for the generation of Abeta, together with evidence of reduced levels in the brain of AD patients of some trafficking related proteins involved in the retromer function (especially the neuronal vesicular protein sorting

10 (VSP10) motif-containing "sortilin-related receptor, L(DLR class) A repeats-containing) protein" (SORL1)[156]) led to pioneering molecular genetic and cell biological studies the author and colleagues to test their hypothesis that genetic variants in genes within the VPS10 sorting proteins might modulate risk for AD[157]. This led to the discovery by the authors group that several clusters of non-coding single nucleotide polymorphisms (SNPs) in the SORL1 gene showed strong association with AD[157]. This association with AD risk, which has now been confirmed in several independent cohorts[158-163], arises from non-coding sequence variants which reduced SORL1 mRNA and protein levels in brain tissue from carriers of AD-risk alleles in SORL1. Further molecular biological work by the author's group[157] and others revealed that SORL1 binds APP, and acts as a sorting switch to direct APP away from late endosomal compartments where Aβ is made. In the relative absence of SORL1, APP is sorted into these Aβ-generating pathways, thereby causing more Aβ to be made[157] (Figure 8). This appears to be the basis through which SORL1 variants are associated with increased risk for AD.

More recently, the author and colleagues have dramatically extended these results by showing also shown that several other members of the VPS10-containing sorting protein family (e.g. SORCS1, SORCS2, SORCS3) are associated with risk for AD[164]. Moreover, as would be predicted, the author and colleagues have been able to demonstrate epistatic (super-additive) genetic interactions between these genes. The underlying mechanism for this interaction appears to be that these genes also modulate APP trafficking in the endocytic-endosomal-retromer pathways and when down regulated they result in increased Aβ production (ref[164] and Reitz et al, submitted).

III.1.a.vi. Late onset AD genes recently uncovered by GWAS methods

By 2009, it was apparent that when taken together, all of the genes described above accounted for only about one third of the overall risk for AD attributable to genetic factors. Much of that risk was accounted for by APOE. Several strategies were applied to identify the remaining AD genes. This included the application of linkage methods in families, sib pair methods, and candidate gene methods. No new genes with robust replication were discovered. Several groups, including the author's, therefore set up a consortium

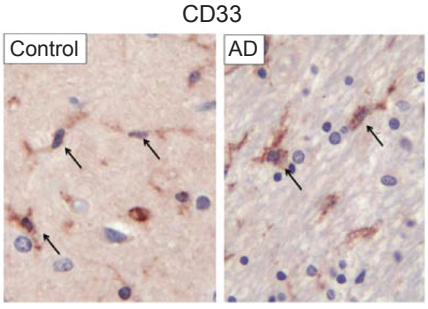
(Alzheimer's Disease Genetics Consortium – ADGC) to apply appropriately powered, large-scale genome-wide association studies (GWAS) employing ≥ 3000 AD cases and ≥ 3000 controls in North West European populations. Using this strategy we have discovered and then replicated modestly strong associations (OR: 0.86-1.2) between late onset sporadic AD with SNPs in the complement receptor (CR1), clusterin (CLU), PICALM, BIN1, ABCA7, CD2AP, CD33, EPHA1, and MS4A genes[165-167].

The discussion below reports recent results from the author's group that has been directed towards identifying the underlying genetic variants in some of these disease genes, and understanding their biological effects. In the next few paragraphs, the author focuses on the description of genes involved in inflammatory pathways the authors group has focused on the innate immune and inflammatory pathway genes because it is clear that these genes depict an important pathway that had previously been overlooked, or at least discounted as an important pathogenic mechanism in AD. Indeed, inflammation had generally been regarded as a late and very secondary complication of neurodegeneration, rather than a driver of neurodegeneration. However, the genetic results described below now make it obvious that innate immune and inflammatory pathways are unexpectedly amongst the primary drivers of the disease, and at the same time are also rich in molecular targets for novel diagnostics and/or therapeutics.

III.1.a.vi.i. Late onset AD genes involved in inflammation

As noted in the previous paragraph, genetic discoveries about the innate immune and inflammatory pathway genes (described in detail below) are extremely important discoveries because these pathways potentially rich intractable diagnostic and therapeutic targets that might be exploited for patient benefit.

Preliminary studies reveal that **CD33** is expressed in microglial cells. In AD cases the CD33-positive microglia are activated (Figure 16). CD33 is a member of a major subfamily of sialic acid binding immunoglobulin like lectins[168]. CD33, like other members of this family have immune receptor tyrosine-based inhibitory motifs and signal negatively to Toll-like receptors during innate immune responses. *CD33* acts as an endocytic receptor, mediat-

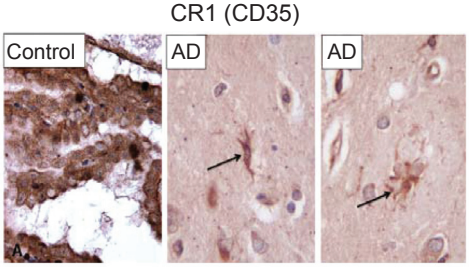


CD33 stains microglial cells (arrows) in both the control and AD cases. In AD cases the CD33-positive microglia are activated as shown by increased cytoplasm.

Figure 16: CD33

ing endocytosis through a mechanism independent of clathrin. CD33 is known to be involved in apoptosis during an immune response[169]. Mutational analysis of this gene is ongoing, but our working hypothesis is that sequence variants in CD33 alter microglial and bone marrow derived macrophage function involved in the uptake and clearance of extracellular Aβ and tau aggregates.

CR1 (complement receptor 1) is a glycoprotein expressed on erythrocytes, leucocytes and follicular dendritic cells, and mediates cellular binding to particles and immune complexes that have activated the complement pathway (especially complement factors C3b and C4b-opsonised foreign antigens). In this role, it mediates immune adherence and phagocytosis. CR1 can also act as a negative regulator of the classic and alternative comple-

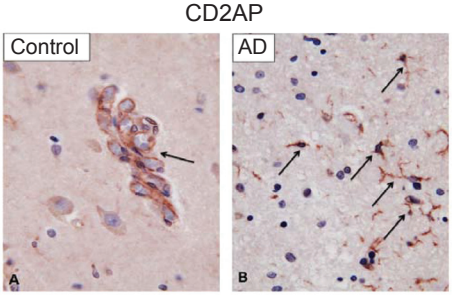


CR1(CD35) stains strongly the choroid plexus in the control and AD cases (not shown). In AD cases, astrocytes (middle) and microglia (right) are also stained.

Figure 17: CR1

ment pathways. CR1 is expressed in the choroid plexus, but not in the brain parenchyma (Figure 17). In AD cases, astrocytes and microglia are strongly stained with CR1 antibodies. Intriguingly, CR1 genotype is associated with the endophenotype of amyloid plaque burden[170]. Our mutational analyses have identified that the pathogenic allele in CR1 is the duplication of a low complexity repeat motif (CR1-S)[171]. This coding sequence insert alters the intracellular processing of the longer isoform, causing it to be retained in the ER, and resulting in less CR1 at the cell surface (unpublished). Consequently, the AD-associated allele in this innate immune/inflammatory pathway gene likely also has a deleterious effect on innate immune/inflammation-mediated removal of extracellular aggregates of Aβ and tau etc.

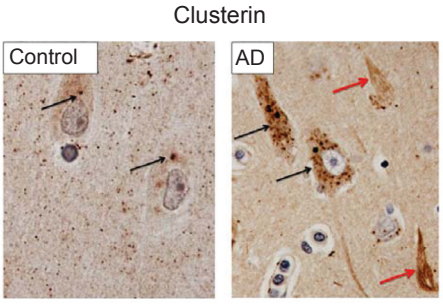
We have shown that **CD2AP** stains vessel walls in control brains, whereas in AD there is also strong microglial labelling (Figure 18). CD2AP is an endocytosis associated protein which interacts with the clathrin scaffold during clathrin-mediated endocytosis. Mutants in CD2AP are associated with nephrotic syndrome. However, CD2AP has also been implicated in dynamic actin remodeling and membrane trafficking/receptor patterning in T-cells and is capable of modulating T-cell receptor signaling[172 173]. CD2AP is also involved in cytotoxic processes by Natural Killer (NK) cells[172]. Mutational analyses are underway. Functional studies suggest that knockdown of CD2AP is associated with impaired microglial activation in the presence of Aβ (manuscript in preparation). These studies again suggest that loss of function alleles in CD2AP result in an impairment of innate immune/inflammation-mediated clearance of neurotoxic extracellular protein aggregates.



CD2AP stains vessel walls in control brains, whereas in AD there is also extensive and strong microglial labeling . The vascular staining is maintained in AD (not shown)

Figure 18: CD2AP

We detected that **CLU** (clusterin) labels small punctate cytoplasmic "inclusions" in neurons. These "inclusions" become more profuse and more numerous in AD cases compare to controls (Figure 19a). In AD cases, CLU also stains plaques and neurofibrillary tangles. Mutational analysis has revealed two types of AD-associated variants. The most common variant is the presence of SNPs in a non-coding intronic regulatory element that regulates protein abundance (presumably by reduced mRNA transcription) (Figure 19b). Work by our colleague David Klenerman, suggests that clusterin is important for chaperoning Aβ and precluding oligomer formation[174]. Lower levels of clusterin might therefore be expected to encourage aggregate formation. The second type of AD-associated variant that we have discovered



Clusterin labels small punctate cytoplasmic "inclusions" in normal neurons in controls. These become more profuse and more numerous in AD cases (black arrows). Clusterin does also stain neurfibrillary tangles (red arrow) and plaques (not shown) in AD cases.

Figure 19a: CLU

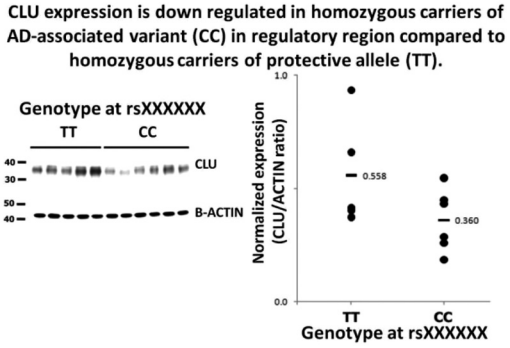


Figure 19b: CLU

are a number of missense mutations and one in-frame nine nucleotide (three codon) deletion in the β-chain of the CLU protein[175]. We are currently investigating the effect of these mutations. However the expectation is that they will also cause a loss of function effect on the chaperone activity of CLU, and like the other AD associated variants will reduce the removal of neurotoxic extracellular protein aggregates.

Finally, during whole exome sequencing of affected pairs of AD cases from families with multiple AD cases ("multiplex"), we identified several families where the affected members had heterozygous missense mutations in the Triggering Receptor Expression On Myeloid cells 2 gene (**TREM2**)[78]. TREM2 is expressed on a number of cells including bone marrow-derived macrophages (microglia) and neurons. We are exploring the effect of these mutations (e.g. R47H), which are probably loss of function alleles. Our working hypothesis is that once again these variants reduce capacity to remove extracellular neurotoxic protein aggregates of Aβ and/or tau.

III.1.a.vi.ii. Transcriptional Micro-Array Profiling of Aβ-Responsive Genes in the Brain of Mouse Models of AD Revealed Early Dysregulation of Genes Involved in Inflammation.

Another method to identify genes involved in disease processes is to use unbiased, genome-wide transcriptional profiling methods (either micro-array-based or RNA-Seq-based methods). We have recently applied the former method to investigate genes that were dysregulated in the brain as a consequence of very early increases in Aβ levels in the brain of our mouse model of AD (TgCRND8 mice-see above). We specifically chose time points that occurred at, or shortly after the first detectable increase in Aβ levels (70-90 days), well prior to any evidence of end organ damage or significant pathology (> 150 days) (Figure 20).

These studies revealed transcriptional dysregulation of multiple genes in innate immune and inflammation pathways (including C3, C4, C1Q and some of the above genes (CLU, CR1, TREM2)(Figure 20). This result, which is entirely congruent with the human genetic data clearly indicated that the dysregulation of innate immune and inflammation pathways is a very early mechanistic event in the pathogenesis of AD. This contrasted strongly with

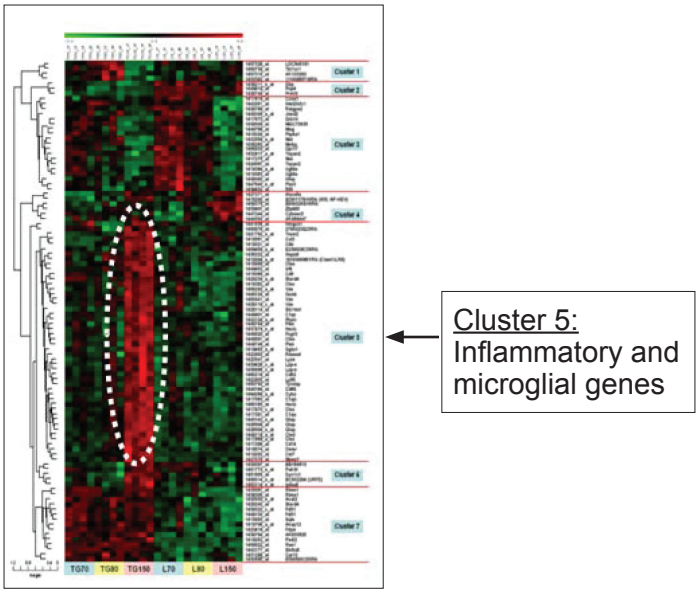


Figure 20: Heatmap of Affymetric microarray analysis of changes in gene transcription in TgCRND8 brain immediately after the first detectable increase in brain Aβ levels (left half) compared to age-matched wild-type control mice. Blue-Green = low expression. Yellow-Red = increased expression. Dotted Circle surrounds a cluster of innate immune and inflammatory pathway genes that are upregulated in TgCRND8 compared to control.

the previously widely-held (but incorrect) view that activation of inflammation in the brain of patients with AD was simply a late consequence of tissue damage.

III.1.a.vii. Combining Genetics, Genomics, to Identify Causal Pathways in AD

Classical *Drosophila* geneticists have long understood that genes which interact together and supra-additively (epistatically) enhance or suppress a phenotype of interest are likely to be genes that function in the same signalling or metabolic pathway. Very often, once such interactions have been identified, a biochemical explanation can be found.

While it is impossible (and unethical) to set up the appropriate cross-breeding studies in humans, careful analysis of cases can nevertheless dem-

onstrate such epistatic interactions. Genome-wide, hypothesis-free screens for epistatic gene: gene interactions can theoretically be done on human GWAS data. However, the number of possible gene:gene interactions that would need to be tested becomes very large when large GWAS SNP screens (>1.2 million SNPs) are investigated. Corrections are needed for false positives due to multiple testing when such large numbers of SNPs are investigated. This requires very large datasets of samples. As a result, the datasets of human cases that can be reasonably acquired to test these interactions usually lack sufficient statistical power for interactions to be sought using a genome-wide hypothesis-free approach. Nevertheless, smaller, more restricted lists of candidate gene:gene interactions can often be generated other sources of information (e.g. looking for gene pairs that are transcriptionally dysregulated at the same time point).

In the next few paragraphs, two examples from the author's work are used to demonstrate the powerful types of information that can be gathered from finding gene: gene interactions using these approaches.

The first example demonstrates that APP and APOE function in the same pathway. Shortly after the discovery of the first mutations in the APP gene, we identified a family from Canada which segregated am APP Val717Ile mutation (Figure 21)[112]. The majority of family members across multiple generations typically developed symptoms of AD at around age 53 years.

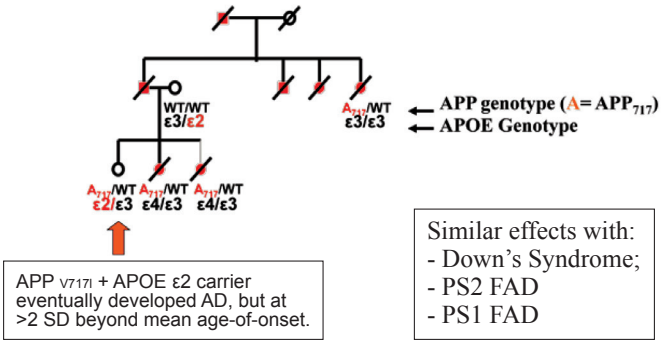


Figure 21: Demonstrating the epistatic interaction between variants in the APP and APOE genes. Most carriers of the APP717 mutation developed AD at approximately 53 years. These individuals also typically had APOE genotypes of ε3 or ε4. In contrast, one carrier of the pathogenic APP 717 mutation inherited a protective ε2 allele from the married-in parent, and had a much later age-of-onset.

However, we identified one family member who carried the same APP mutation, but who remained asymptomatic for more than 15 years beyond the mean age of onset for that family (> 2 standard deviations beyond mean). This family lived in a small, remote, close-knit rural community. There was no evidence for significant environmental diversity amongst family members. However, we observed that the majority of carriers of the APP mutation had APOE ε3 or ε4 (Figure 21)[118].

In sharp contrast, the family member with the considerably delayed onset of symptoms had inherited a protective APOE ε2 allele from their married-in parent (Figure 21).

This key observation indicated that there had been a genetic suppressor interaction between alleles at APOE and APP, and strongly suggested that these two genes interacted in the same metabolic/signalling pathway. Subsequent studies revealed similar modulator interactions between genotypes at APOE alleles and APP, PS1 and PS2. These studies are therefore clearly indicated that these genes cause AD through influencing APP processing and the accumulation of neurotoxic Aβ[118]. This concept was subsequently proven biochemically by showing that APOE binds Aβ, and that PS1/PS2 are components of the γ-secretase enzyme which cleaves APP to generate Aβ (Figure 8).

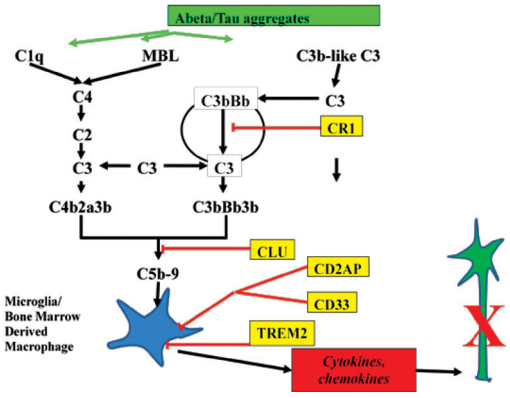


Figure 22: Several genes identified by the GWAS studies as having variants associated with risk for AD are known components of innate immune and inflammation pathways. Variants in these genes are likely to modulate the severity of inflammatory responses to protein aggregates. Inadequate inflammatory response, or over-exuberant inflammatory response to Aβ/tau aggregates could cause chronic neuronal injury.

A second set of biochemical and genetic interactions were subsequently discovered between several VPS10-containing vesicular trafficking proteins such as SORCS1 and SORCS3 (Reitz et al, submitted 2012). All of these genes alter risk for AD by changing APP processing and altering the amount of Aβ produced (Figure 8). Finally, as described in the section immediately above, evidence of a third set of interactions is emerging between several genes involved in innate immunity and inflammation pathways (Figure 22). In contrast to the other two examples, genes in this pathway appear to modulate risk of AD by affecting the responses to Aβ oligomer accumulation (either reduced clearance and/or increased neurotoxic inflammation).

III.1.a.viii. Conclusions from Genetics, Genomics, Cellular and Molecular Biology, Animal Modelling of AD

Cumulatively, the analysis of the data arising from transformative experiments in genetics, genomics, cellular and molecular biology and animal modelling of Alzheimer's disease are beginning to sketch-out the systems biology of AD (Figure 23).

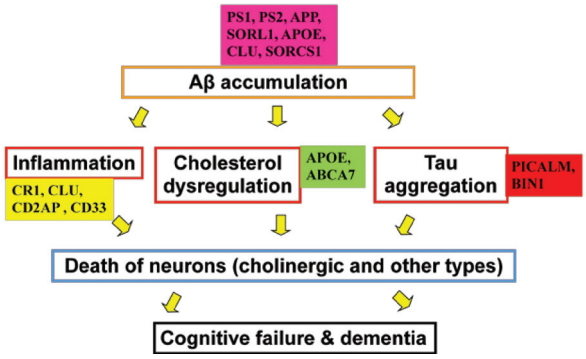


Figure 23: The application of molecular genetic, genomic, cellular and molecular biology, and animal modelling shows that the multiple different genes associated with AD risk can affect inter-linked pathways involved in the production and clearance of Aβ, and lead to the accumulation of Aβ in the brain. There are also genetic variants in several downstream pathways involved in inflammation, cholesterol and lipid metabolism, tau aggregation and spreading. It is likely that these variants govern the activity of those downstream pathways. Many of these pathways may be self propagating once activated. Greater knowledge of their nodal control points and systems biology will facilitate the discovery of: 1) novel diagnostic and theragnostic biomarkers; and 2) novel molecular targets for new therapies against these pathways that react to this accumulation of Aβ peptide.

The picture is beginning to emerge that there are multiple genetic variants which alter risk for AD by modulating the production of Aβ (magenta box in Figure 23). These genes affect Aβ levels by altering the sequence of APP, by altering its subcellular trafficking and distribution, and by modulating its proteolytic cleavage into peptide fragments, some of which (Aβ) are potentially neurotoxic.

It is also becoming increasingly clear that there are genetic variants in a handful of other genes involved in pathways regulating innate immunity, inflammation, cholesterol and lipid metabolism, uptake and re-secretion of protein aggregates ("intraneuronal spreading"). The genetic variants in these genes likely modulate risk for AD by altering the flux through downstream pathways in response to a given load of Aβ.

The results described above have truly transformed our understanding of the biology of Alzheimer's disease. More importantly, they generate targets that can be further developed as diagnostic or theragnostic biomarkers, and as molecular targets for novel therapies.

III.2. Fronto-Temporal Dementia Adisorders

In contrast to AD, where the genetic factors imply a common biochemical pathogenesis (accumulation of neurotoxic Aβ oligomers), frontotemporal lobar degeneration (FTLD) is a clinically, biochemically, neuropathologically and aetiologically heterogeneous syndrome. It represents a group of primary degenerative dementias with predominant frontal and/or temporal lobe symptoms (e.g. decline in social and personal behavior, apraxia, stereotyped behavior, hyperorality and aphasia)[176,177]. The clinical nosology of the FTLDs is complex and includes clinically-defined classifications such as behavioural variant FTD (bvFTD), Primary Progressive Aphasia (PPA), Posterior Cortical Atrophy (PCA), frontotemporal lobar degeneration with motor neuron disease (FTLD-MND), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP) and FTD with parkinsonism linked to chromosome 17 (FTDP17).

The neuropathological characteristics of FTLD are also complex and correlate only weakly with the clinical characteristics. All FTLD cases have gross frontal and temporal lobe atrophy with neuronal loss and gliosis

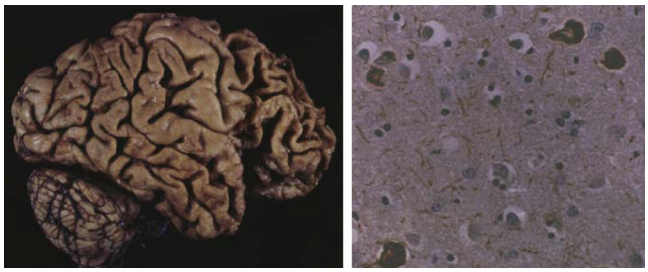


Figure 24: Fronto-temporal atrophy (left) with neuronal tau inclusions (right) in a case of FTD-Tau.

(Figure 24). However, the microscopic pathology varies markedly in different forms of FTLD. Thus the deposition and/or abnormal processing of the tau protein can be seen in ~40% of cases. This type of tau-pathology is typical of cases with mutations in the tau gene (see below). However, up to 60% of FTD cases lack MAPT-positive neuronal inclusions, displaying mainly microvacuolization of the superficial neuropil in the cortex (often with ubiquitin-positive inclusions in cortical neurons). Up to 50% of these tau-negative cases contain small, ubiquitin-positive, Tar DNA Binding Protein 43 (TDBP43) positive inclusions[14,178] (Figure 25). These cases often have mutations in progranulin, TDP-43 itself or C9ORF73 gene expansions (see below). A smaller proportion of non-tau FTLD cases display Fused in Sarcoma (FUS) deposits (often due to mutations in FUS itself – see below). The remaining cases have relatively non-specific pathology, often with minor spongiform changes plus or minus intra-neuronal ubiquitin deposits.

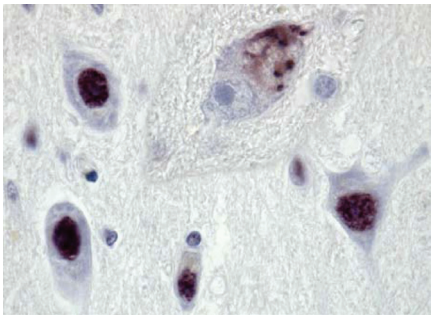


Figure 25: Ubiquitinated TDP43 deposits in the cytoplasm of cortical neuron of a case with progranulin mutation described by the author's group.

III.2.a. MAPT (tau) gene:

The microtubule-associated protein tau, encoded by the MAPT gene is important for microtubule assembly and stabilization. Tau is normally expressed in axons, but in FTD (and AD) it is redistributed to the cell body and dendrites. In the normal adult human brain, tau protein is soluble and exists as six isoforms generated by alternative splicing. Splicing of exon 10 controls the number of microtubule binding domains, generating either so-called 3-repeat or 4-repeat tau (Figure 26).

To date, at least 45 pathogenic MAPT mutations have been reported (age-at-onset 20-80 years) (http://www.molgen.ua.ac.be/ADMutations/) by various groups[179,180], including the author's group (Figure 26)[181]. Some MAPT mutations cause a phenotype resembling progressive supranuclear palsy (R5L and G303V) or FTD with motor neuron disease (K317M). The majority of MAPT coding sequence missense mutations are clustered in exons 9-13, and at least some of these mutations may alter binding of tau to microtubules (reviewed in [176]). However, some of the exon 10 missense substitutions and the intronic mutations affect the alternative splicing of exon 10 and cause an increase in 4-repeat tau.

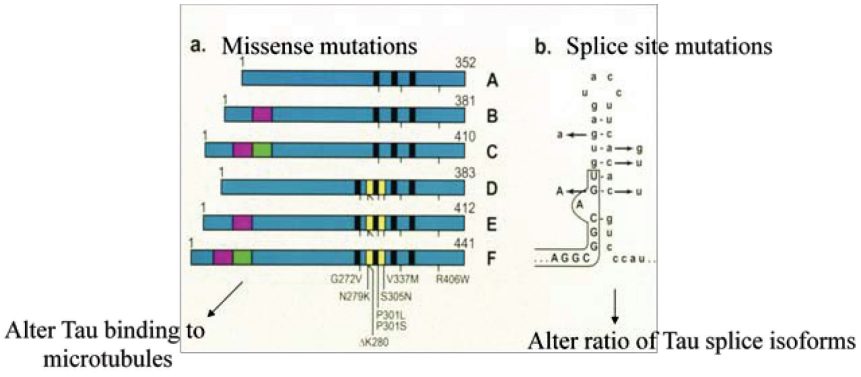


Figure 26: Both missense mutations and splice site mutations in the tau gene are known to underlie inherited autosomal dominant tauopathies causing neurodegeneration. Missense mutations have clustered around the microtubule binding repeats. Other mutations have occurred in the splice site at exon 10, and alter the relative abundance of splice forms containing three microtubule binding repeats versus those which contained four microtubule binding repeats. Both types of mutation appear to alters the binding of tau to microtubules.

Progressive Supranuclear Palsy (PSP) is frequently included amongst the tau-based overlapping syndromes of FTD, PPA, CBD and FTDP-17. However its genetics is more complex and is therefore separately described in greater detail in Section III.4 below.

III.2.b. Progranulin (PGRN) gene

At least 250 families have been described by various groups[182,183] including the author's group[184] in which mutations in the progranulin (GRN) gene located on chromosome 17q21 cause autosomal dominant FTD. Potentially-pathogenic mutations in the GRN gene have also been reported in AD, ALS, and PD patients. This raises the possibility that progranulin may contribute to other neurodegenerative diseases. Patients with GRN mutations do not have tau-pathology. Instead there are ubiquitin-immunoreactive neuronal cytoplasmic and intranuclear inclusions which stain for the TAR DNA Binding Protein 43 (TDP43)[14].

GRN encodes progranulin, a secreted glycoprotein growth factor which seems to be involved in the regulation of multiple processes including development, wound repair, inflammation and maintenance of neurons during aging. Progranulin protein binds to the sortilin receptor, which mediates its internalization and lysosomal degradation.

The FTLD-causing mutations have typically been loss-of-function (typically missense or truncating) mutations. The mutant missense or truncated transcripts are destroyed by nonsense-mediated decay, resulting in partial loss of GRN protein. It is presently unclear how partial loss of GRN expression leads to neurodegeneration[184].

The functional relationship between GRN and TDP43 is presently unclear. However, progranulin can be measured in the plasma, CSF and serum, and patients with pathogenic mutations in progranulin have lower levels, which can be used as a screening test for this form of FTLD[185,186].

Importantly, a number of drug-like compounds have been found which can increase GRN gene transcription. The observations that mutation carriers can be easily screened for, and that the effect of GRN mutations is to lower GRN transcription now raises the possibility that plasma progranulin screening could identify patients at-risk for the disease. Once identified,

these patients could then be treated with compounds to increase GRN levels to normal. Clinical trials are underway.

If successful, FTLD due to GRN mutations would then become an easily preventable cause of dementia – like the use of penicillamine for the chelation of copper in subjects at-risk for Wilson's disease.

III.2.c. C9ORF72

Genetic linkage studies in families with FTD + MND or with ALS defined a common region of 3.7 Mb on 9p21[187-194]. Concomitantly, GWAS studies of sporadic ALS or FTD also suggested a major risk factor located at the same region, which contains three known genes *(MOBKL2B, IFNK, and C9ORF72)*[195-199]. Subsequent sequencing studies of these candidate genes identified a disease-associated expansion of a non-coding GGGGCC hexanucleotide repeat in the promoter of the C9ORF72 gene as the underlying molecular defect/mutation[200-202]. This repeat expansion co-segregates perfectly with diseases in families with FTD + MND or ALS, and appears to be a frequent cause of these familial disorders, accounting for 46.0% of familial ALS, 21.1% of sporadic ALS and 29.3% of sporadic FTD.

The molecular mechanism by which this expansion causes disease, is currently under investigation. However, there is approximately 50% reduction of C9ORF72 mRNA expression in carriers[200-202], leading to the hypothesis that the hexanucleotide expansion causes a loss of function effect, much like the effect of many progranulin mutations. Carriers have nuclear RNA granule inclusions that are frequently associated with TDP43 aggregates in the cytoplasm of neurons, suggesting that the expansion might alter the processing of either RNA itself, and/or RNA Binding Protein metabolism. If correct, the pathogenic mechanisms underlying C9ORF72-associated forms of FTD and ALS might be similar to the mechanisms induced by mutations in FUS and TDP-43[200-202].

Additional studies are currently underway to determine the mechanism underlying why the disease phenotype is so variable (FTD, MND or both), even within the same family segregating an expansion in the *C9ORF72* gene. Possibilities include the existence of significant modifier genes (e.g. progranulin, FUS, TDP 43, tau) or variability in the expansion size itself.

Indeed, it is currently unclear why the same mutation can in some individuals cause ALS and in others FTD.

Expansions in the *C9ORF72* gene should be considered in patients with a strong family history of either ALS or FTD + MND where expansions in C9ORF72 may account for up to 40% of FTLD-MND families.

III.2.d. Other Rare Genetic Causes of FTLD

Several rare forms of FTLD have been discovered. These are described briefly below. They illustrate the biological heterogeneity of FTLD.

Dynactin 1 (DCTN1) gene:

DCTN1 protein binds directly to microtubules and is critical for neuronal function. Recently, DCTN1 mutations were reported in very rare patients with slowly progressive, autosomal dominant form of lower motor neuron disease without sensory symptoms, Amyotrophic Lateral Sclerosis (ALS) (M571T and T1249I) and ALS with FTD (R1101K)[203].

Charged Multivesicular Body/ Chromatin-modifying protein 2B (CHMP2B) gene:

Recently, CHMP2B mutations resulting in aberrant mRNA splicing were reported to be the cause of autosomal dominant FTD or ALS in a very small number of families (including a large Danish family). CHMP2B maps to chromosome 3p12.1[204]. The brains of affected individuals show global cortical and central atrophy, but no Aβ-amyloid or tau deposits are found.

Valosin-containing protein (VCP) gene:

Inclusion body myopathy associated with Paget disease of the bone and FTD (IBMPFD) is rare an autosomal dominant disorder that maps to chromosome 9p21.1-p12[205]. Up to 40% of patients manifest cognitive decline with predominant frontal lobe features in the later clinical phase (after 50 years of age). At least six different missense mutations clustering in the ubiquitin-binding domain of the VCP gene have been identified in IBMPFD families[205]. VCP is a multifunctional protein associated with nuclear envelope reconstruction, Golgi reassembly, DNA damage response, apoptosis

suppression and ubiquitin-dependent protein degradation. The IBMPFD-associated mutations cause neuronal nuclear inclusions (containing VCP and ubiquitin) and VCP aggregates in scattered muscle fibers[205].

Intraflagellar Transport 74 (IFT74)

Recently, a Q342X truncating nonsense mutation and a G58D biochemically significantly missense mutation were detected by the author's group and colleagues in highly conserved residues of the Intraflagella Transport 74 (IFT74) gene on chromosome 9 families with ALS-FTD[206]. The biological nature of these mutations suggests that they are likely to be pathogenic. As was the case with progranulin and tau genes on chromosome 17q, there may also be a second FTD gene on chromosome 9 because no mutations were seen in the IFT74 gene in several other pedigrees showing genetic linkage to another nearby region of chromosome 9.

Familial British Dementia (FBD) and Familial Encephalopathy with Neuronal Dementia with Neuroserpin deposits

Recently, two very rare forms of inherited dementia (Familial British/Danish Dementia and Familial Encephalopathy with Neuroserpin Inclusions (FEN1b)) have been described which also support the emerging concept that some adult-onset dementias are disorders in which there is either intracellular or extracellular accumulation of toxic misfolded/misprocessed proteins.

Familial British Dementia and Familial Danish Dementia are allelic variants of the same disorder, and are characterized by spasticity, ataxia, and later by progressive dementia and widespread demyelination with distinctive perivascular fibrous deposits that are clearly different from the plaques of AD. The British variant is caused by T->A transversion mutation in the stop codon of the Integral Membrane Protein 2b (ITM2B) gene (also known as the BR1 gene) on chr 13 which causes the addition of several amino acids at the C-terminus of the BRI protein[207]. In the Danish variant, there is a 10-bp duplication one codon before the stop codon. While the normal function of the BRI protein is unknown, the presence of the extra C-terminal amino acids induced by both mutants causes the protein to be misprocessed by a Furin-mediated cleavage. This results in the accumulation of a 34 amino acid

C-terminal derivative termed the ABRI peptide that assembles into toxic amyloid deposits through mechanisms which remain to be elucidated[208].

Missense mutations in Neuroserpin - a neuron specific serine protease inhibitor (serpin) have been described in two pedigrees with a familial dementia[209]. In these families with Familial Encephalopathy with Neuroserpin inclusion Bodies (FEN1B) the mutant neuroserpin forms typical serpin loop-sheet polymers that assemble into fibrous aggregates that appear as 5-50 μm PAS-positive eosinophilic inclusions called "Collins bodies" in the deep layers of the cerebral cortex, subcortical nuclei and substantia nigra.

III.3. Prion Protein (PRNP) Gene

Up to 15% of cases with prion-related diseases are due to the autosomal dominant inheritance of mutations within the PRNP gene on chromosome 20pter-p12 (http://www.mad-cow.org/prion_point_mutations.html). Inherited forms of prion diseases include Creutzfeldt-Jakob disease, Gerstmann-Straeussler disease and Fatal Familial Insomnia, which present with clinically variable phenotypes including, ataxia, myoclonus and rapidly progressive dementia. Some PRNP polymorphisms modify the disease phenotype. An interaction between the common M129V polymorphism and the pathological D178N mutation might result in either Creutzfeldt-Jakob disease (V129-N178 allele) or Fatal Familial Insomnia (M129-N178 allele). Furthermore, homozygosity for either M129 or V129 is increased in patients with iatrogenic forms of CJD acquired during surgical procedures, etc., and in the variant form of CJD. The exact explanation is not yet proven, but may relate to more efficient recruitment of homo-monomers into PrP aggregates and fibrils (see review[210]).

III.4. Progressive Supranuclear Palsy

Progressive Supranuclear Palsy (PSP) is a rare neurodegenerative movement disorder first characterized by the Toronto neurologists Richardson, Steele and Olszewski. PSP is characterized clinically by rigidity, bradykinesia, frequent falls, vertical supranuclear gaze palsy and cognitive decline. It is often confused with Parkinson's disease, but differs from Parkinson's disease in the absence of a response to dopamine replacement. Pathologically,

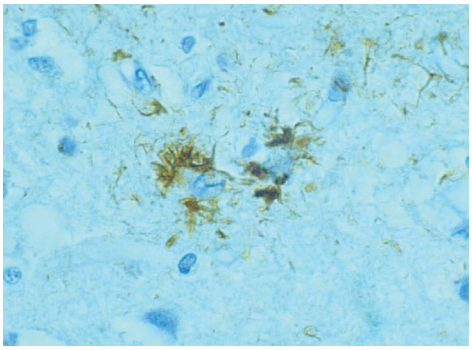


Figure 27: Tau-positive inclusions in a "tufted astrocyte" from a PSP case

PSP is characterized by the presence of aggregates of tau as neurofibrillary tangles in neurons from various parts of the brain, including the basal ganglia, forebrain and brainstem. Tau aggregates also accumulate in oligodendroglia and astrocytes where they give rise to the appearance of "tufted astrocytes" (Figure 27). The aetiology of PSP is likely complex with both environment and genetic factors.

We[211] and others[212] were amongst the first to show that specific haplotypes of the tau gene (specifically the H1 haplotype) were associated with increased risk for PSP. Intriguingly, this H1 haplotype also contributes to risk for corticobasal degeneration, the Guam ALS-Parkinson-Dementia Complex., and Parkinson's disease. The association with PD may be a confounder due to inclusion of PSP cases within the PD cohort, or alternatively due to an effect of the H1 haplotype on the risk of cognitive impairment in PD patients.

However, although the H1 haplotype in tau is a strong risk factor for PSP, it does not account for all of the attributable genetic risk for PSP. Consequently, in collaboration with others, we undertook a genome-wide association study (GWAS) that included over 2,200 cases of PSP and over 6,700 controls[213]. This led to the discovery for the first time that risk for PSP was also associated with variants in the syntaxin 6 (STX6), the eukaryotic translation initiation factor 2 – alpha-kinase 3 (EIF2AK3) and the myelin-associated oligodendrocyte basic protein (MOBP) genes. The association with STX6 is reminiscent of the associations of several other neuro-

degenerative diseases with genes involved in vesicular protein trafficking (e.g. SORL1 in AD as previously discovered by the author, CHMP2b in FTD, etc.). Similarly, the association with EIF2AK3 gene, which is involved in the endoplasmic reticulum unfolded protein response (UPR), is in good agreement with the notion that many neurodegenerative disorders arise from intracellular or extracellular protein misfolding (e.g. as demonstrated by the author and by others, AD is associated with CLU, a molecular chaperone of Aβ). Finally, the association with MOBP initially led to the intriguing concept that there might be significant involvement of white matter in PSP. However, subsequent work by the author's group (unpublished) suggests that the non-coding variants in the MOBP gene in fact regulate the expression of a nearby gene which is involved in neuronal apoptosis. Significantly, this nearby gene is massively upregulated in the brain of patients with PSP (unpublished). Because this gene encodes a receptor or channel protein, and because PSP-associated SNPs in this gene result in its over-expression in PSP, the discovery of suitable small-molecule blockers might provide a novel therapy for PSP.

IV
Clinically Useful Applications of the Products of Basic Molecular Research on Neurodegenerative Diseases

As described above, in the last two decades there have been truly monumental gains in our understanding of the pathobiology of neurodegenerative dementias. Knowledge about these diseases has grown from simple clinical and pathological descriptions, to an increasingly detailed understanding of the molecular mechanisms underlying many of these neurodegenerative dementias. This change in our knowledge has in no small part been due to the genetic discoveries described above. This improved understanding has been so profound that it is about to cause a huge paradigm shift in some very practical and clinically useful ways in how these diseases will be diagnosed and treated in the future.

Specifically, this new knowledge has brought a better understanding of the clinical course of these diseases. These diseases are now known to have a much longer preclinical asymptomatic period had been expected ($\geq$ 15 years)[4,5]. This unexpectedly long preclinical phase provides unparalleled opportunity for early diagnosis and treatment. The emerging knowledge about the genes causing these diseases has permitted the development of tractable, albeit still imperfect, model systems such as transgenic mice[49] and cultured neuronal systems developed from induced pluripotent stem cells[214]. The emerging knowledge has led to the development of useful biomarkers based in: cognitive neuroscience (e.g. the Paired Associative Learning Test, which accurately predicts conversion from Mild Cognitive Impairment to florid AD[215]); biochemistry (e.g. CSF tau and Aβ assays[216-218]); and neuroimaging (e.g. structural and functional MRI and positron emission tomography scans for CNS amyloid and inflammation). These and other future biomarkers will help with early detection of the disease and with monitoring the effects of therapy. Finally, this emerging knowledge has encouraged the development of treatments designed at disease-modification, rather than symptomatic management. There are now several trials of first generation therapies directed at modulating Aβ accumulation in AD. There are attempts to identify compounds that will address the mislocalisation, accumulation and aggregation of tau in AD, PSP and FTLD-tau. The discovery that progranulin levels are reduced in carriers of GRN gene mutations has raised the possibility that progranulin levels can be returned towards normal using small molecule compounds that activate GRN gene transcription. The discovery that inflammation is an important and early part of pathogenesis of many neuro-

degenerative diseases has pushed forward attempts to identify appropriate biomarkers and therapies for neuroinflammation.

Two decades ago, these ideas were unthinkable.

In the following pages, the current manuscript will use examples from the author's own work to illustrate a few of these dramatic advances in translating fundamental (basic) science discoveries into clinically applicable products for patient benefit.

IV.1. Useful Knowledge about The *Pre*-Clinical Course Of Neurodegenerative Diseases

Studies of the genetic forms of neurodegenerative dementias have generated hitherto unknown facts about the course of these diseases.

Until recently, there was no firm knowledge about the preclinical stage of these diseases. It was literally unknown when these diseases actually began. It could have been a year before onset of symptoms. It could equally have been a decade before. However, by using longitudinal follow-up studies of asymptomatic carriers of known disease-causing mutations, it has now become quite clear that, with the exception of prion-related diseases, nearly all of the neurodegenerative diseases have very long pre-symptomatic periods. Thus, following carriers of mutations in the PS1 gene by the author and by other groups, has led to the understanding that there are biochemically easily detectable changes in amyloid and tau metabolism that occur 10-15 years before the onset of any detectable symptoms[5 4,5]. This knowledge is critical because it defines a clinically-important window in which the application of sensitive preclinical biomarkers may detect cases. Individuals in this preclinical biochemical phase of the illness can be treated before irreversible neuronal damage has occurred. Such insight was impossible before the discovery of the relevant disease-causing gene (e.g. PS1)[6,153, 4,5].

IV.2. Clinically Relevant Use in Generating Informative Animal Models

There are no naturally occurring animal models of the human neurodegenerative dementias. Many useful mechanistic experiments can be done in

simple biochemical and cultured cellular systems (e.g. monitoring cleavage of APP to generate Aβ peptide). However, there are many aspects of the disease which require models that mimic the long slow progressive nature of the human disorders. Consequently, in the absence of natural models, a relatively early step was to use the mutant disease-causing genes to create transgenic models in invertebrate and vertebrate animals. As would be expected from the complex nature of these diseases and from the innate differences between humans and nonhuman animals, few, if any, of these transgenic animal models perfectly replicate ***all*** aspects of the human disease. Nevertheless, when the results of the experiments are interpreted appropriately and conservatively, these model organisms do provide very robust systems for investigating selected aspects of these diseases (e.g. the effect of Aβ accumulation in APP transgenic mice; the effect of FUS aggregation on neurons in transgenic *C. elegans* models, etc.). To illustrate the utility of different model organisms, two models generated by the author's group are described below.

IV.2.a. Invertebrate models: an example of invertebrate FTLD/MND-FUS model

As noted above, a significant proportion of FTLD/MND cases arise either from primary mutations in RNA binding proteins such as FUS and TDP-43, or due to misprocessing of these proteins because of aging, unknown environmental factors or mutations in other genes (e.g. GRN, C9ORF72, etc). We have therefore generated transgenic models of FTLD-FUS in *C. elegans* by expressing either wild-type or mutant human FUS under the neuron-specific *Prgef-1* promoter[219] (Figure 28).

Key phenotypes in this *C. elegans* model of FTLD-FUS replicate several key features of FUS-induced disease in humans. First, animals expressing *wild-type* human FUS have normal intra-cellular distribution of FUS and no neurophysiological phenotype[219]. In contrast, human *C. elegans* animals expressing *mutant* human FUS undergo age-dependent aggregation of FUS in the cytoplasm of neurons, together with progressive motor deficits and accelerated mortality[219]. These features closely resemble those of human patients with FUS mutations. Second, the aggregates in neurons of humans

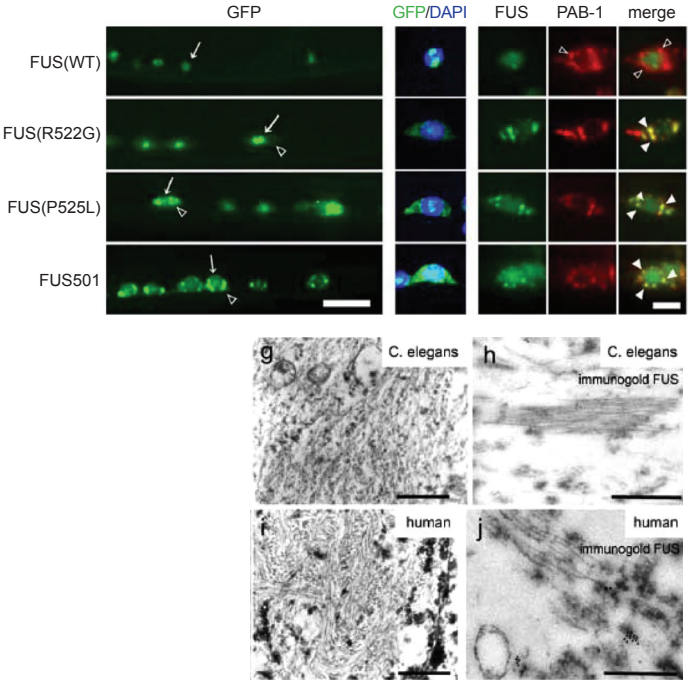


Figure 28: **Upper panel:** In *C. elegans* expressing *wild type* human FUS, FUS is expressed in neuronal nuclei (normal). In mutant FUS expressing animals the *mutant* FUS aggregates in the cytoplasm. Green = GFP-tagged FUS; Blue = DAPI nuclear stain.
Lower panel: Identical structural EM (left) and immune-EM (right) properties of mutant FUS aggregates in *C elegans* neurons (g, h) and in neurons from human FTLD case.

and in neurons of the *C elegans* model possess similar morphology and biochemistry. By electron microscopy, intraneuronal FUS aggregates in human neurons and in *C. elegans* neurons are composed of a mixture of 10-20nm coated and non-coated fibrils and granular material. Biochemically, FUS aggregates in human neurons and in *C. elegans* neurons can be solubilised in 2% SDS, and they do not stain with amyloidophilic dyes such as Thioflavin S. Finally, the severity of the neuronal dysfunction induced in *C. elegans* by a given FUS mutant, closely parallels the severity of the clinical illness in humans with the same mutation[219].

We used this model to discover two previously unrecognized features of FUS- and TDP43-dependent neurodegenerative dementias. First, exist-

ing work had suggested that because these proteins were RNA-binding proteins, the effect of disease-causing mutations was to alter cellular RNA processing. We showed that the pathogenic mechanism was misfolding into neurotoxic aggregates. Second, we showed that misfolding of mutant FUS induces a previously unrecognized form of neurotoxic protein aggregate. This novel type of protein aggregate differs biophysically from traditional neurotoxic amyloid proteins (e.g. Aβ, tau). These novel aggregates are soluble in 2% SDS and do not stain with Thioflavin S. These hydrogel proteins are normally able to reversibly aggregate on cytoplasmic Stress Granules. The mutations cause a conformational shift in the hydrogel protein which causes the aggregation to become both irreversible and neurotoxic.

We have also used this model to show that small molecule aggregation inhibitors like scyllo-inositol can inhibit FUS misfolding, aggregation and neurotoxicity.

This highly innovative invertebrate model therefore demonstrated, for the first time, a novel disease mechanism. The model also provided critical proof-of-principal for a novel therapy for FTLD-FUS. The novel concepts of hydrogel cytotoxic aggregates and their use as a therapeutic target will be equally applicable to many other diseases caused by other hydrogel-forming proteins including TDP-43 and TAF15 mutations, which also cause FTLD.

IV.2.b. Vertebrate Models: An Example of Mouse Model of Amyloid Pathology of AD

The absence of naturally occurring animal models of human Alzheimer's disease (AD) has been a major limitation both to understanding the pathobiology of AD, and to the efficient preclinical testing of novel diagnostics and therapeutics. As a result, the development of tractable artificial animal models has been a desperately sought-after goal for the last two decades. Initial attempts at developing transgenic models based upon overexpression of APP were unsatisfactory[220-222]. It was only in the mid-1990s that mice were developed with sufficient overexpression of mutant human APP transgenes to generate believable AD like neuropathology[49,223,224].

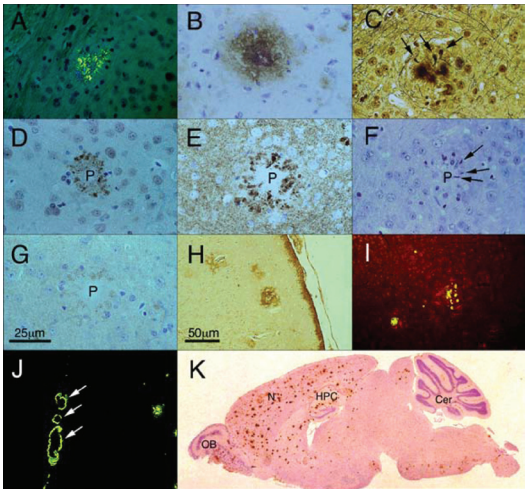


Figure 29: TgCRND8 mouse model of AD developed by the author. This mouse displays many of the amyloid-dependent features of human AD including congophilic dense cored amyloid plaques, diffuse amyloid plaques in the neocortex and hippocampus, phosphorylated tau-positive neuritic dystrophy, perivascular congophilic amyloid angiopathy. These features are associated with synaptic injury and loss of synapses, and by progressive cognitive impairment.

One of these robust transgenic murine models of AD is the TgCRND8 mouse developed by the author and colleagues (Figure 29). This model has now been widely and freely used by academic and commercial members of the research community[49]. The TgCRND8 mouse expresses a human amyloid precursor protein transgene *(APP_{695})* bearing two missense mutations that cause AD in humans (KM670/671NL and V717F). Beginning at about three months of age, these mice display progressive spatial learning deficits that are accompanied by rising cerebral Aβ levels and by increasing numbers of cerebral amyloid plaques[49,225]. By six months of age, the concentration of Aβ and the morphology, density and distribution of amyloid plaques are similar to those seen in brains of humans with well-established AD[49,225]. In addition to the clear-cut amyloid pathology, there is also significant activation of inflammation as manifest by the appearance of increasing numbers of activated microglia and astrocytes, and by up-regulation of transcription of inflammation pathway genes on RNA profiling studies. Finally, there is progressive synaptic loss as demonstrated by the reduction in staining for presynaptic and postsynaptic proteins such as synaptophysin and PSD-95

respectively. As in human patients with AD, these biochemical, behavioural and neuropathological phenotypes are accompanied by accelerated mortality[49,225].

This mouse model has been catalytic for both basic science studies of the pathogenesis of AD, and also for preclinical translational experiments of novel therapies. It has been used in over 230 published reports that have been cited more than 4300 times (H Index = 31).

As an example of its utility in basic science studies, TgCRND8 helped elucidate the functional relationship between APP and the presenilin genes (PS1/PS2). The presenilins are now known to encode the catalytic subunit of γ-secretase, and are responsible for the last step in the generation of Aβ (see Figures 8, 13, 14). However, even before the true function of the presenilin proteins was understood, by creating transgenic mice over-expressing mutant PS1 proteins, and crossbreeding them to TgCRND8, we discovered that was a massive (i.e. multiplicative rather than additive) increase in the pathology of the double transgenic mice (Figure 30). This unequivocally told us that mutations in PS1 and APP acted in the same metabolic/signalling pathways. We were subsequently able to prove biochemically that both proteins do indeed function in the same pathway. The presenilins are the enzyme that cuts APP to generate Aβ.

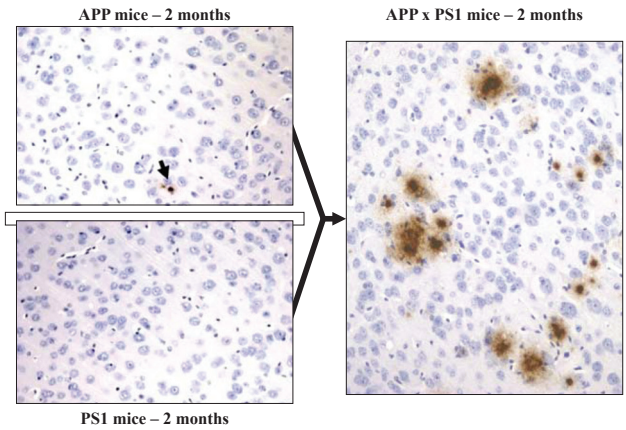


Figure 30: Supra-additive interaction between APP and PS1 : double Tg mice (right) have a more the 10-fold increase in Aβ pathology than do either single Tg line (left), confirming that APP and PS1 are in the same pathway. It was subsequently shown that APP is the substrate for PS1-mediate γ-secretase activity.

Equally transformative was the ability to use these mice in preclinical proof-of-principle experiments for novel therapies (See Section IV.4 below and Figures 33-36).

We initially used these mice in pioneering studies to demonstrate that immunotherapy against Aβ peptide could significantly improve the amyloid-dependent features of AD (cognitive impairment, amyloid plaque load)[49]. We then used these mice to show that residues 4-10 on Aβ constituted the major immune epitopes on Aβ that were recognized by therapeutically effective antibodies (residues 4-10)[226]. Finally, we used this animal model in proof-of-principle experiments to demonstrate that the small molecule Aβ-aggregation inhibitor, scyllo-inositol, was a potential therapeutic for AD[50]. This pioneered a novel therapeutic strategy for AD, and suggested that anti-aggregants might be useful in other neurodegenerative diseases as well.

Many other academic and commercial groups have exploited these mice for preclinical testing of novel compounds that inhibit inflammation, Aβ aggregation, γ-secretase or β-secretase activities. Many of the candidate therapeutics tested and refined on our TgCRND8 mouse model have then proceeded on to human clinical trials. Examples include: scyllo-inositol[227], anti-Aβ vaccines such as Bapineuzumab[228-231]; and novel (unpublished) β-secretase inhibitors developed by Merck/Schering-Plough.

IV.3. Use of Molecular Genetic Clues for Novel Diagnostics of Dementias

The discovery of mutations in genes causing neurodegenerative diseases (e.g. APP, PS1, tau, TDP-43) led to the understanding that these mutations caused biochemical alterations in the encoded protein. It rapidly became apparent that functional changes in the mutant protein could be detected in post-mortem human tissues (as amyloid plaques (APP, PS1); neurofibrillary tangles (tau); and skein-like intraneuronal deposits (TDP-43)). This correlation between mutation, functional changes in the encoded protein and neuropathologically-detectable changes in postmortem brain immediately encouraged efforts to detect biomarkers of the misprocessing these proteins in plasma, CSF, and brain of living patients. The area of biochemical and neuroimaging biomarkers (Figure 31) is well dealt with in many recent re-

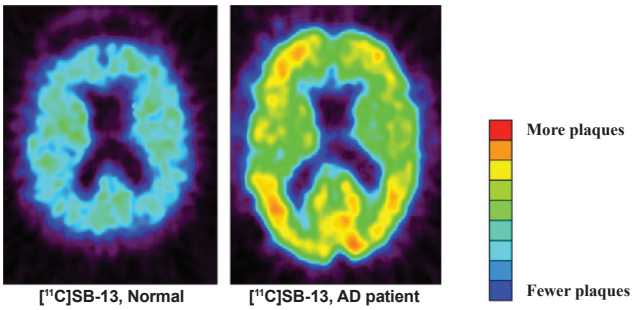


Figure 31: Amyloid-binding PET ligand ^{11}C-SB-13 similar to PIB developed in Toronto, showing significant uptake in the cortex of an AD patient.

views, and will therefore not be further summarized here. A less obvious, but predictably, increasingly important concept is the idea of using genetic tests in the management of patients with neurodegenerative dementias. This concept is dealt with in greater detail. This analysis of the emergence of genetic tests draws upon both the work of the author, and upon the work of other experts.

IV.3.a Genetic Tests for Diagnosis of Specific Neurodegenerative Dementias

Presently, the use of genetic tests in the management of patients with neurodegenerative disease is relatively limited. In most instances it is confined to the issues surrounding genetic counselling of members of families with clear-cut autosomal dominant patterns of inheritance (see below).

However, as outlined below, this situation is likely to change dramatically in the coming years. This change in the implementation of gene-based tests is likely to be coupled with three emerging trends.

First, there is growing awareness that the classical clinical nosology of these diseases is inadequate. Patients in the same family with the same mutation can have quite different clinical phenotypes. This is especially the case with some of the FTLD/MND-causing genes. The use of gene-based tests will dramatically facilitate achieving the correct molecular diagnosis and coupling it with the appropriate disease modifying therapy.

Second, it is anticipated that new disease-modifying therapies will be developed that are targeted to specific aspects of the pathogenesis of these diseases (e.g. Aβ production, inflammation, tau aggregation). It will be important to ensure that patients, especially at-risk patients, are provided with the most appropriate of these targeted disease-modifying therapies. This can be done by identifying patients with disease-associated variants in genes involved in each of these treatable aspects of the disease (e.g. variants in complement receptor 1 (CR1) might regulate the degree of inflammation induced by Aβ, and might require specific anti-inflammatory therapy).

Third, and directly related to this, there is increasing interest worldwide in the use of personalised medicine strategies. The central hypothesis of personalized medicine is that the therapeutic regime should be tailored to the genotype of individual patients (e.g. as in the example above, the early use of anti-inflammatory therapies in individuals with genetic variants in disease-associated genes in inflammation pathways - such as Complement Receptor 1 (CR1).

The types of applications for genetic tests in the management of patients with neurodegenerative disease are therefore described in greater detail below. It is understood that there are significant ethical and legal issues surrounding the use of genetic tests in incurable neurodegenerative diseases, but these issues are well understood (see review in[232]).

IV.3.b Genetic Tests for Diagnosis of in Symptomatic *Cases*

Even in the absence of effective therapies or preventions, the ability to apply a genetic diagnosis to symptomatic cases with familial dementia often improves the physician's ability to provide mechanistic explanation for the disease, and to forecast the likely clinical course of the disease for both the patient and his/her caregivers. This aspect of genetic testing is especially useful when the disease-causing gene is highly penetrant, and where there is a sharp differentiation between non-disease-causing polymorphisms and disease-causing mutations. Under these circumstances, with genes like PS1, PS2, APP, tau, etc. the discovery of a pathogenic mutation can be diagnostically very helpful.

Typical examples of when access to this genetic information is particularly productive are when the clinical features of affected family members are atypical, or where the illness is pleomorphic, with different family members having slightly different phenotypes. Examples of the latter are members of families with certain PS1 mutations where the presentation can occasionally resemble Frontotemporal dementia or spastic paraparesis (cotton wool plaques).

Another example is the highly pleomorphic nature of the FTLD/MND phenotype in some families with mutations in FUS, TDP-43 or C9ORF72. Some family members may have a predominantly Frontotemporal dementia syndrome, while others may have a predominantly motor neuron disease phenotype. Molecular genetic tests are now available for all of the known dementia-causing genes on a gene-by-gene basis from academic and commercial testing labs. In the near future, cost-effective chip-based diagnostics containing SNPs and INDELs in all of the genes associated with dementia will be available (i.e. the "NeuroChip"). This will allow comprehensive molecular diagnosis. Ultimately, whole exome or whole genome-based sequencing can be envisaged as the preferred diagnostic method. By allowing screening of the entire genome, these emerging methods permit diagnosis of both novel and already-known mutations causing these diseases.

The question of whether genotypes at incompletely penetrant dementia-related genes (e.g. APOE, CLU, CR1, progranulin, etc.) are diagnostically useful in symptomatic cases is more nuanced. In some of these genes (APOE) not all carriers of the disease associated polymorphisms will develop symptoms of the disease in their lifetime. In the case of APOE, whether the APOE genotype may be of assistance in establishing the diagnosis of AD in demented patients undergoing diagnostic work-up is also a matter of some discussion. Most experts agree that while the APOE genotype studies might form a part of the diagnostic armamentarium, APOE genotype is not the only test that should be done even in individuals with classical clinical features of AD[233]. Thus, Mayeux and colleagues have shown that a clinical diagnosis of AD has a sensitivity of ~93% and specificity of 55%, whereas the APOE ε4 allele confers sensitivity and specificity of 65% and 68% percent, respectively. The addition of information about the APOE genotype increased the overall specificity to 84% in patients who met the clinical criteria for Alzheimer's disease, but the

sensitivity decreased 61%. However, it is important to note that the patients studied in the report by Mayeux et al were referred to tertiary centres, and it remains to be determined whether genotype would provide similar levels of sensitivity and specificity in more typical clinical settings.

The problem is even more complicated for many of the other genes where there are many disease-associated non-coding and coding variants as well as large numbers of non-pathogenic non-coding and coding variants. Deciding which variants (coding or non-coding) are pathogenic is often very difficult, and great care is needed about making/communicating clinical predictions and diagnoses based on such ambiguous information. Fortunately, in the case of progranulin variants, there is a relatively easy way of distinguishing pathogenic mutations from polymorphisms. The pathogenic GRN mutations generally cause missense mediated RNA decay, and therefore lower plasma progranulin levels[185,186]. It is possible that plasma clusterin values might also predict which AD patients have CLU variants[234,235].

IV.3.c. Genetic Tests for Predictive Genetic Testing in currently **asymptomatic** *first-degree family members*

The discovery that mutations in specific genes are associated with inheritable susceptibility to dementia, leads to the frequent need for physicians to consider the merits of genetic counselling and genetic testing in currently non-symptomatic family members. At the present time, in the absence of clearly effective preventative or curative treatments the main reason for genetic counselling and testing is to provide information only. While such information can be empowering, it obviously also has the potential to be misused to the patient's disadvantage, and must therefore be handled carefully. However, once effective therapies become available, even relatively imprecise estimates of genetic risk can be very useful in identifying and preemptively treating even subjects who are only mildly at-risk subjects.

There is currently little practical experience with genetic counselling of members of families multiply affected with AD or the other dementias discussed here. Consequently, most of the paradigms used for genetic counselling of members of families with dementia are based upon similar paradigms used in the counselling of subjects with Huntington's disease[236]. The Huntington's

disease model is actually quite useful for counselling of members of families with autosomal dominant, early-onset familial Alzheimer's disease (FAD) associated with mutations in PS1, PS2 or APP and of FTD associated with mutations in Tau, FUS, TDP43, progranulin and perhaps C9ORF72 because the age-of-onset is often similar (30-65 years of age) and has a similar pattern of transmission (highly penetrant, age-dependent penetrance, autosomal-dominant segregation). Thus, in members of families with mutations in the APP, PS1 and Tau genes, it is possible to screen at-risk family members for the presence of mutations detected in affected individuals, and to counsel these family members based upon the concept that APP, PS1 and tau mutations are highly penetrant (approximately 95%) with typical age-of-symptom-onset between 35 and 65 years. Screening for PS1 mutations is also cost-effective when done on symptomatic cases with a positive family history of AD and onset before the age of 60 years[237]. PS2 mutations on the other hand have a lower penetrance and a more variable age-of-onset (45-85 years).

Mutations in genes causing highly-penetrant, autosomal dominant dementias (e.g., PS1, tau, progranulin, TDP43, FUS, C9ORF72 and APP) are a comparatively rare cause of familial dementia. Cumulatively, they account for about 50% of early-onset FAD (which itself accounts for ~5% of all AD), and 10-40% of familial Frontotemporal dementia.

A much more common clinical experience is the presence of two or three affected family members with late-onset dementia in a small nuclear pedigree. Frequently in these "multiplex" families, the disease does not inherit as a classic autosomal-dominant trait. As a result, in any given family, it is frequently unclear whether the multiplex pedigree structure reflects an incompletely penetrant autosomal-dominant trait, or a more complex mode of transmission involving either the interaction of several genes or the interaction of genes and environment.

Empirical counselling of asymptomatic members of pedigrees of this type is difficult, and must largely be based upon recent epidemiological studies. While the use of molecular genetic studies would clearly facilitate counselling in such pedigrees, the only locus for which there are significant amounts of data in multiple populations is the apolipoprotein E (APOE) gene. Retrospective studies, in autopsy and clinical series, have suggested that the cumulative lifetime risk for AD in subjects homozygous for APOE

ε4 may be as high as 90% by age 90 years. However, even from these retrospective studies, it is apparent that there is a huge variation in the age-of-onset of AD even in subjects who are homozygous for APOE ε4 (50-90 years). To confound matters further, a small number of limited prospective studies suggest that the APOE genotype is a relatively poor predictor of the onset of AD even in high risk groups such as those with age-associated memory loss[238]. Consequently, a number of research groups have recommended that the APOE genotype not be used for presymptomatic testing because of concern about adverse effects and misunderstanding of the true risk associated with low penetrance alleles[239]. However, a recent long-term prospective study (REVEAL) of asymptomatic, at-risk individuals who have been genotyped with APOE[240] demonstrated that although the subjects' recollection of the magnitude of their risk was often wrong, knowledge of the APOE genotype was not associated with psychological risks[241].

IV.3.d. Genetic Tests for Diagnosis: Can genetic tests be used if the disease gene is unknown?

It is apparent, from the discussion above on the molecular genetics of AD and related disorders, that a significant proportion of familially-aggregated dementia cannot be related to polymorphisms or mutations in any of the known genes. It is likely that in the next several years, additional AD and FTD susceptibility genes will be identified. However, until most or all of these genes are identified, and their relative frequencies as a cause of AD or FTD in specific populations can be defined, genetic testing of at-risk family members can only be reasonably performed if there is a testable, clinically-affected member available. If an affected pedigree member is available, their DNA can be screened for mutations in the known susceptibility genes. If mutations are found, this information can then be used for the testing in at-risk family members. Where no mutations in the affected subject's DNA are found, it can be reasonably assumed that the disease is caused by mutations or polymorphisms in other AD/FTD genes yet to be identified (or the disease is not genetically specified), and mutation screening studies in at-risk family members would not be indicated. However, in the absence of a living affected member who can be tested, the screening for mutations in the DNA

of at-risk family members is currently likely to be a fruitless task because, until all of the disease-causing genes for that trait are known, the failure to discover disease-related polymorphisms or mutations in the known genes can give no reassurance that the at-risk family member is not a carrier of a mutation in another susceptibility gene.

IV.3.e. Genetic Tests for Experimental Medicine Drug Trials and In "Personalised Medicine" Approaches to Dementia Therapy

Another role of genetic testing is in the design of a therapeutic program for subjects affected with AD and other dementias ("personalised medicine"). Given the obvious genetic heterogeneity of AD (and the other dementias), it is conceivable that some subtypes of AD (or subtypes of the other dementias) might respond better to specific therapeutic agents than others. For instance, it is reasonable to suspect that individuals with AD-associated variants in genes involved in inflammation and innate immunity (i.e. CR1, CD2AP, CD33, CLU) may have defects in innate immune clearance of neurotoxic protein aggregates, and/or defects in the inflammatory response to these aggregates. Targeting immune and inflammatory pathways with suitable anti-inflammatory treatments might be more effective in individuals with variants in these genes, than in individuals with the same disease, but with variants in other disease associated pathways.

An important corollary has emerged to this concept. There is a growing consensus of opinion that clinical trials can be made faster and more cost effective if small genotypically-focussed cohorts of experimental subjects can be assembled based on the presence of risk alleles in known disease-causing genes that are the target of the candidate therapeutic. For instance, as above, CR1 variant carriers might be used to test novel for anti-inflammatory drugs.

These "Experimental Medicine" trials can be coupled with biomarker endpoints in that same pathway (e.g. in this example, a reasonable theragnostic biomarker might be PET neuroimaging of microglial activation). Modelling studies suggest that proof-of-principal/proof-of-mechanism can be obtained in these "Experimental Medicine" trials with small cohorts of subjects (n=30) in short time frames (≤ 6 motnhs) and with considerable cost savings.

IV.4. Using the Knowledge of Emerging From Basic Science to Identify Novel Therapeutic Targets and Design New Disease-Modifying Treatments

As described above, the basic science work performed by the author and many other groups over the last several decades has helped to dissect the molecular mechanisms underlying neurodegenerative dementias.

For most of these diseases, it is becoming increasingly obvious that the molecular mechanisms involve a complicated series of interconnected pathways. For instance, in AD there is compelling evidence that Aβ accumulation directly induces synaptotoxicity. However, it is increasingly clear that Aβ also indirectly induces neuronal injury through several downstream events including: 1) misprocessing of tau; 2) alterations in cholesterol and lipid metabolism; and 3) activation of inflammation. Many of these secondary processes (e.g. tau aggregation; inflammation) are both neurotoxic and self-propagating (viz. "inflammation begets more inflammation").

This concept of an initial event with multiple downstream effector pathways implies that anti-Aβ monotherapy may only work in the very early stages of the disease. Once the other downstream processes are activated and become self-propagating, anti-Aβ therapies will need to be supplemented by specific therapies directed at these specific secondary pathways. The connections between amyloid, tau, and inflammation are clearly far more complex than originally envisaged by the jingoistic term "amyloid cascade".

A major goal for the next decade will be to understand the systems biology of these complex pathways. It will be important to identify the key nodal control points that govern rate-limiting steps within these pathways. These control points might provide foci for the creation of novel biomarkers (to measure the flux through that node) and therapeutics (to block flux through that node). However, even though we do not yet have a *complete* knowledge of the systems biology of these pathways, the knowledge that has already emerged in the last decade clearly gives glimpses of potentially tractable therapeutic targets. These glimpses of potential mechanism-based therapies are most advanced for AD. Consequently, in this manuscript, the author will focus on recent attempts to translate the emerging basic science knowledge about AD into potential disease-modifying treatments for this disease.

IV.4.a. From Basic Science to Candidate Disease-Modifying Therapies for AD

The powerful genetic evidence indicting the accumulation of Aβ peptide as a central event in the pathogenesis of AD has encouraged a huge amount of effort to identify tractable therapeutic targets within Aβ-generating and Aß-clearance pathways. A significant effort has been specifically directed towards the development of both small molecule therapies and biological therapies. These mechanism-based therapies have been designed to: block the accumulation of Aβ (e.g. with small molecule β- or γ-secretase inhibitors); block the aggregation of Aβ (e.g. with small molecule inhibitors like scyllo-inositol); or accelerate the removal of Aβ (e.g. with anti-Aβ vaccines) (Figure 32).

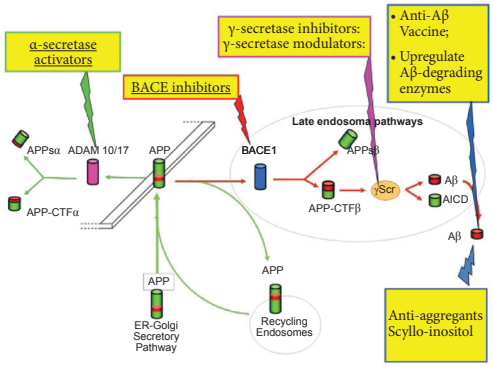


Figure 32: Potential therapeutic targets within Aβ-generating and clearance pathways.

Because of the increasing understanding of the important role of tau aggregation in AD, there are significant parallel efforts underway to develop compounds that will inhibit the phosphorylation and aggregation of tau. Anti-tau vaccines are being developed and tested in preclinical models of cerebral tauopathy. These vaccines might prevent inter- neuronal spreading of tau. Mirror image projects are also underway to develop similar antibody strategies to prevent α-alpha synuclein spreading in Alzheimer variants with α-synuclein deposits (which are present in about 30% of FAD cases due to PS1 mutations[4,242,243]), in Dementia with Lewy Bodies (DLB) and in Parkinson Disease with Dementia.

The emerging data from human molecular genetic and from animal model studies suggest an early and important role for the innate immune system and inflammatory pathways in the pathogenesis of AD (see above). Consequently, a variety of anti-inflammatory and anti-complement therapies are being considered.

The following paragraphs describe work done by the author, which illustrate the approaches involved in translating emerging basic science knowledge into early Experimental Medicine trials in humans. Both of the projects described below have proceeded into full-scale human clinical trials.

IV.4.a.i. Immunotherapies

Basic science studies on the mechanisms of aggregation of Aβ peptide revealed that epitopes at the N-terminus of the Aβ peptide played a key role in this process. Antibodies directed against the N-terminus of Aβ were capable of disaggregating preformed amyloid fibrils, and preventing the formation of new fibrils.[244] Induction of anti-Aβ antibodies in the PDAPP mouse revealed that was a small proportion of plasma anti-Aβ antibodies did cross the blood brain barrier, and were capable of inducing significant clearance of amyloid plaques, and reducing total brain Aβ load in this mouse model. However, it was unclear whether this reduction in brain Aβ pathology was accompanied by any cognitive improvement. Slowing or reversal of cognitive decline is clearly the *sine qua non* for useful therapies for AD. Simple removal of Aβ without functional cognitive benefit would have little value. To resolve this question, the author's group undertook the key experiment to determine whether or not antibody-mediated removal of Aβ had any effect on cognitive function in the TgCRND8 mouse model of AD[49].

These sentinel experiments by the authors group revealed unequivocally that, when given before the onset of symptoms, active immunization against Aβ peptide was capable of significantly reducing both: 1) the neuropathological and biochemical accumulation of Aβ (Figure 33); and 2) the extent of cognitive impairment in these mice (Figure 34)[49]. Significantly, and perhaps under-appreciated at the time, we pointed out that this clearance was better when done presymptomatically, and had only an incomplete effect when done in symptomatic TgCRND8 mice[49].

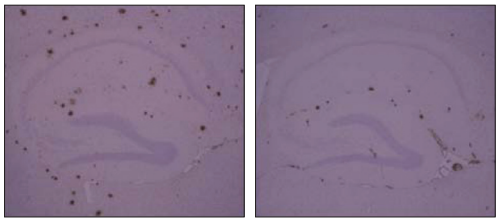


Figure 33: Active anti-Aβ immunisation of TgCRND8 mice also results in dramatic reductions in numbers of amyloid plaques, amount of information, and improves the number of cortical synapses. However there is little change in the total Aβ load, suggesting that the antibodies address a very specific, small subpopulation of Aβ species (perhaps soluble oligomers). Similar results have now been seen with some anti-Aβ trials in humans. The explanation why anti-Aβ immunisation has only worked partially or not at all in humans may reside in which Aβ species the antibodies are directed at.

The author's group then followed-up these sentinel experiments by subsequently using a novel mass spectrometry approach to identify the immune epitope on the Aβ peptide domain that was recognized by therapeutically effective antibodies[226]. We showed that these antibodies recognized residues 4-10 of Aβ. However, these antibodies also showed marked conformational specificity, they recognized only oligomeric and fibrillar aggregates, but not monomeric Aβ peptide[226].

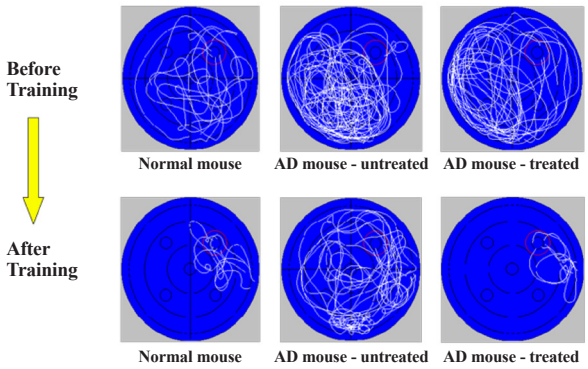


Figure 34: Morris water maze tests of the cognitive behaviour of normal and AD (TgCRND8) mice before (untreated) and after anti-Aβ vaccination (treated). Prior to training normal mice would swim randomly in the maze. Following training, the normal mice would remember the location of the hidden underwater platform, and would swim directly (left panels). Unvaccinated TgCRND8 (middle panels) would swim randomly both before and after training, being unable to remember the location of the hidden platform. In contrast, anti-Aβ vaccinated mice (right panels) showed essentially normal spatial memory, and were able to learn the location of the hidden platform.

These experimental results were part of the preclinical body of evidence which underpinned the decision by multiple major pharmaceutical and biotechnology companies (Elan, Pfizer, Lilly, Genetech, Roche) to develop passive and active vaccines against Aβ peptide. This same collection of work also encouraged the subsequent idea to test similar immunotherapies against other cerebral protein aggregates including tau[245,246] and alpha synuclein[247]. The equivalent mouse studies done by other groups replicated our study design and have suggested possible benefit. Clinical trials are now being considered for anti-tau and anti-α-synuclein immunotherapies as well[245-248].

IV.4.a.ii. Small molecule aggregation inhibitors: scyllo-inositol from bench to bedside

As noted above, multiple lines of evidence suggest that the accumulation of neurotoxic oligomeric aggregates of amyloid β-peptide (Aβ) may be a central event in the pathogenesis of AD[36]. If correct, this hypothesis predicts that inhibitors of Aβ-aggregation and toxicity may effective in blocking this pathogenic cascade. Prior data from several groups had demonstrated that Aβ-oligomerization and fibril formation is strongly facilitated by phosphatidylinositol lipids in cell membranes. We therefore hypothesized that derivatives of these phosphatidylinositol moieties, particularly myo-inositol stereoisomers (which also act as osmolyte modulators of protein folding[249]), might compete with intact phosphatidylinositol for binding to Aβ and interfere with Aβ fibril assembly. In vitro proof-of-principle experiments by the author's colleagues indeed revealed that myo-inositol and scyllo-inositol (Figure 35) can inhibit Aβ fibril assembly; accelerate disassembly of

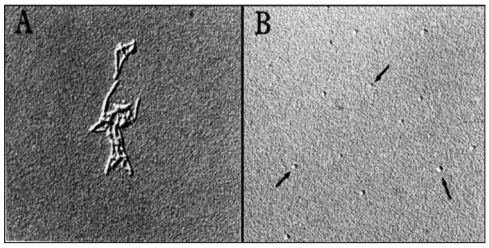


Figure 35: Aβ assembles into amyloid fibrils (panel A), but scyllo-inositol causes Aβ to assemble into non-toxic globules (panel B).

preformed fibrils; preferentially stabilize Aβ into non-toxic β-structured spherical micelle conformers; and protect primary cultured neurons from Aβ-oligomer induced toxicity[250].

To assess the *in vivo* effectiveness of this anti-aggregation approach, we then administered scyllo-inositol to our TgCRND8 transgenic mouse model of AD (described above). We used two different treatment paradigms. In the first paradigm, scyllo-inositol was orally administered as a *"prophylactic",* with treatment beginning at six weeks of age (i.e. ~six weeks prior to the typical age of onset of the AD-like phenotype in TgCRND8) and continuing until either four or six months of age (when the AD-like phenotype is florid). In the second paradigm, compounds were given as a *"therapeutic"* beginning at five months of age (when the AD-like phenotype is already well established), and continuing until six months of age. Within each of these experimental arms, mice were randomly assigned to receive active compound, mock therapy (mannitol, a sugar of similar molecular weight), or no therapy. In both experiments, scyllo-inositol improved cognitive function (as measured by spatial reference learning in the Morris Water Maze test); reduced brain Aβ levels; reduced brain Aβ oligomer levels (as measured by A11 immnuoreactivity); reduced cerebral plaque loads; reduced inflammation and microglial and astrocytic activation; increased synapse numbers as measured by synaptophysin staining; and reduced mortality (Figure 36)[50].

Scyllo-inositol conforms well to the Lipinski 'rule-of- five' for the ideal properties of a drug-like compound[251]. Scyllo-inositol is also transported into the CNS by facilitated transport mechanisms, and has high bioavailability in the CNS. Consequently, scyllo-inositol was taken through pharmacokinetic and pharmacodynamic studies; administration-distribution; metabolism-excretion and toxicity studies in preclinical models (dogs and rats), and then into Phase 1 studies in man. It has recently completed Phase 2 studies in man and a decision is being made on the design of Phase 3 trials[227].

Detailed reviews on the worldwide efforts on a variety of therapeutic targets for Alzheimer's disease are available elsewhere[252,253]. However, one theme that has emerged is that many of the early clinical trials were performed prematurely. This is especially the case for most of the first generation anti-Aβ therapies in almost all of the clinical trials of these first generation anti-Aβ therapies have had to be discontinued, often without clear answers as to

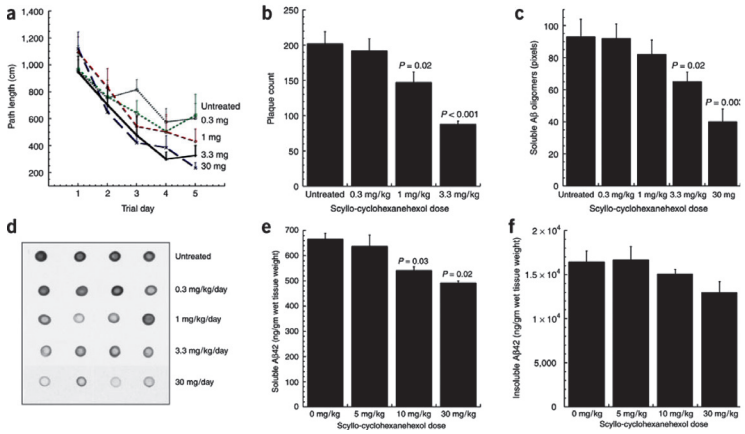


Figure 36: When given prophylactically, Scylloinositol caused a dose-dependent inhibitionin: a) spatial memory dysfunction; b) number of amyloid plaques; c and d) soluble Aβ oligomers as measured by dot blot immuno-reactivity with the A11 anti-oligomer antibody; and e and f) total soluble and insoluble Aβ. Similar , but less profound improvements were obtained when Scyllo-inositol was given after the AD-like phenotype was well established.

whether there was adequate brain penetration, whether the therapeutic target was engaged, and whether there was biomarker effect on the target (even if no clinical change was scored). These premature discontinuations have arisen because of technical problems such as: toxicity of the compound (semagacestat, AN1792 active anti-Aβ immunisation); poor brain penetration of the compound (Tramiprosate); administration of the therapy to cases with very advanced disease (bapineuzumab); and/or poor study design (Alzhemed and several others). The resulting high rate of failure has led to negative statements, particularly in the lay press, that anti-amyloid therapies do not work. In fact, most experts believe that the clinical trials to date have simply not adequately tested the amyloid hypothesis (see the review[254]). Indeed, it is clearly premature to abandon anti-Aβ therapies because there is some tantalising evidence emerging from the recently completed Phase 3 trial of the Lilly anti-Aβ antibody solanezumab. This recently emerging data suggests that solanezumab may well have worked in the very mild AD subgroup (http://www.alzforum.org/new/detail.asp?id=3288). This observation supports the recent notion that anti-Aβ therapies may be best targeted at the very early stages of the disease. As a consequence, there are now plans for a prophylactic treatment trial of presymptomatic carriers of PS1 mutation carriers (see above).

V
Contributions and Discoveries by the Author

The work summarized above clearly depicts a group of highly complicated human brain disorders that could not possibly be solved by any single individual. However, that said, the author's contributions to that body of knowledge have been monumental.

Dr. Peter St George-Hyslop's research has been primarily directed toward elucidating the mechanisms causing human neurodegenerative disease, with research into the molecular basis for Alzheimer Disease constituting the main focus of this work. His laboratory discovered that Alzheimer's disease is etiologically heterogeneous, an observation that subsequently had a profound effect on the design of both clinical and basic research paradigms on this disease. His laboratory directly led to the discovery of multiple genes associated with AD, including presenilin 1, presenilin 2, nicastrin, and SORL1. He co-led in the discovery of two other genes, the amyloid precursor protein with J. Gusella, and apolipoprotein E with A. Roses. More recently, as a member of the Alzheimer's Disease Genetics Consortium, he used Genome Wide Association Study (GWAS) methods in the largest study of AD genetics to that time, to identify at least 9 novel AD genes associated with late onset AD.

Dr. St George-Hyslop's laboratory has shown that: aberrant APP processing and accumulation of the Aβ peptide fragment is central to the pathogenesis of AD; that cerebral Aβ deposition is the earliest pathological feature in presymptomatic cases of familial AD; and that over-production of Aβ is a consequence of AD-causing clinical mutations in several AD genes including PS1 (in collaboration with D. Selkoe and M Citron) and SORL1. Using genetic methods, his laboratory has shown that several of the known AD genes have additive effects and are thus likely to function within the same metabolic or signalling pathway. One of these pathways relates to APP processing and Aβ peptide accumulation and aggregation into a neurotoxic oligomers. However, St George-Hyslop's work also implicates, amongst others, pathways involved in innate immune and inflammation responses to protein aggregates. Using biochemical, cellular, and molecular biological methods, his laboratory has begun to decipher the molecular mechanisms by which genetic variants in these genes and pathways cause AD.

One particular area of his research that arose from his work on AD and the presenilin proteins has turned out to have major implications for a previ-

ously unrecognised basic biological process - Regulated Intramembranous Proteolysis". Thus, he has shown that the presenilins, nicastrin, PEN-2 and APH-1 proteins are components of a tetrameric protein complex (presenilin complex /γ-secretase complex). This complex is the catalytic unit that performs the intramembranous γ- and ε-secretase cleavages of the transmembrane domains of APP and Notch to generate Aβ and Notch Intra-Cellular Domain respectively. This process has been termed "Regulated Intramembranous Proteolysis". He has identified a new regulatory component of the presenilin complexes - TMP21. TMP21 differentially regulates γ- and ε-site secretase cleavage activities. To work out structural mechanics of this unusual enzyme complex, St George-Hyslop has used single particle electron microscopy to generate a 15Å resolution 3D model of the PS1 complex in the presence or absence of small molecule inhibitors. Cumulatively, St George-Hyslop's scientific contributions in this area have provided otherwise unattainable insights into a novel form of protein processing that is essential both for several critical physiological signal transduction events (Notch signaling), and for a key event mechanism underlying AD (Aβ generation). Selective modulation of the presenilin-mediated production of Aβ while sparing Notch signalling remains a candidate therapeutic approach in the treatment of AD.

Work in Dr. St George-Hyslop's group has led to the generation of several superb transgenic mouse models for Alzheimer Disease and Fronto-Temporal Lobar Degenerations. He has used the Alzheimer transgenic mice (TgCRND8) to show that immunization with Aβ reduces both neuropathological and cognitive deficits in these mice. This result has suggested that Aβ immunization, as well as other therapies directed at Aβ biology, may have utility as mechanism-based, disease-modifying treatments and/or preventions of Alzheimer's Disease in humans. This idea has recently received support from some human trials, which have suggested partial benefit to patients with early/mild AD. Moreover, he has defined both the immune epitope in Aβ that is targeted by therapeutically effective Aβ-vaccination. This information will serve as a basis both for improved antigens and for the generation of novel compounds to mimic the effects of antigen:antibody binding. Indeed, he and his team have found a promising series of compounds epitomized by scyllo-inositol, that inhibit Aβ oligomer assembly and toxicity in

preclinical animal models of AD. This compound has recently completed Phase 2 clinical trials in humans.

In addition to his work on AD, St George-Hyslop has made major contributions to the molecular genetics and molecular neurobiology of Fronto-Temporal Lobar Degeneration/Motor Neuron Disease (FTLD/MND). He has discovered novel pathogenic variants in progranulin. He has developed tractable *C. elegans* models of FUS- and TDP-43-dependent forms of FTLD/MND. He has used these models to show that FUS and TDP-43 mutations cause FTLD/MND by inducing these proteins to aggregate into a noveltype of neurotoxic protein aggregate – namely hydrogel-derived aggregates. These aggregates differ from traditional amyloid aggregates in several important biophysical parameters. He has shown that the effects of SOD1 mutations in Familial ALS/MND likely arise from a gain-of-toxic function effect that is independent of SOD1 catalytic activity, and that the copper chaperone for SOD1 (CCS) gene is not the site of mutations causing ALS in families lacking SOD1 mutations.

He has also made contributions to the genetics of other neurodegenerative diseases including Spinocerebellar Ataxias, Dystonia/Parkinsonism, cortico-basal Ganglionic degeneration, Benign Hereditary Chorea and most recently Progressive Supranuclear Palsy, where he has used GWAS methods to identify tau and several new genes involved in vesicular trafficking and innate immunity as risk factors for PSP.

The impact of the author's work can also be assessed by looking at his publications record. The author's publications are difficult to track because his surname can be tracked only with difficulty (viz. Hyslop, George Hyslop, St George-Hyslop, etc.). Nevertheless, his body of work, which is almost exclusively ***primary research*** papers generating new knowledge rather than reviews of other people's work, has been cited more than 27,000 times. His work is cited on average 900 times per year. Several of his papers have been cited more than 500 times, three of them more than 1,000 times, and two of them more than 2,400 times.

The impact of the author's work can also be assessed enumerating the awards and lectures given to him by his scientific peers. These are enumerated in his CV. However, it is worth noting here that he has been elected as a Fellow of the prestigious Royal Society of London (one of the oldest

academic societies in the world), the Royal Society of Canada, and as a Foreign Member of the Institute of Medicine of the US National Academies of Science. It is highly unusual for individuals to be nominated to the elite scientific societies of one country, never mind three! In addition, he has been awarded both of the key international prizes for research in neurodegenerative diseases - namely the Potamkin Prize from the American Academy of Neurology and the Metropolitan Life Award for Medical Research. These peer-driven awards reflect the esteem with which the author's colleagues regard him.

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Grande Prémio Bial de Medicina 2012

Instituído em 1984 pela FUNDAÇÃO BIAL, o PRÉMIO BIAL é considerado um dos maiores prémios na área da Saúde em toda a Europa, distinguindo profissionais de referência mundial nas suas áreas de investigação.

A obra "Translating Discoveries in Basic Molecular Biology, Cell Biology, and Molecular Genetics into Transformative Approaches to the Diagnosis and Treatment of Currently Incurable Neurodegenerative Dementias", de autoria de Peter St. George Hyslop, Diretor do Centro para a Investigação de Doenças Neurodegenerativas da Universidade de Toronto e Professor de Neurociências Experimentais na Universidade de Cambridge, foi galardoada com o GRANDE PRÉMIO BIAL DE MEDICINA.

O PRÉMIO BIAL DE MEDICINA CLÍNICA distinguiu o trabalho "Diabetic Retinopathy. New Perspectives for Personalized Management" de José Cunha Vaz, professor emérito da Universidade de Coimbra e presidente da AIBILI - Associação para a Investigação Biomédica e Inovação em Luz e Imagem.

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O júri da edição PRÉMIO BIAL 2012 foi presidido por António de Sousa Pereira e constituído por Miguel Castelo-Branco, Maria João Marques Gomes, Adelino Leite Moreira, António Martins da Silva, Luís Providência, Nuno Sousa e Rui Victorino.

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