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Alzheimer's Disease: Approaches to Pathogenesis in the Genomic Age

Greg T. Sutherland and Jillian J. Kril
Disciplines of Pathology and Medicine,
Sydney Medical School,
University of Sydney,
Australia

1. Introduction

The prevalence of Alzheimer's disease (AD), the most common form of dementia, is rising rapidly worldwide. A seemingly imminent epidemic is predicted to have wide reaching societal and economic consequences, becoming the leading health issue for many countries by the middle of this century. This epidemic is driven by an ageing world population in combination with a lack of disease modifying treatments or preventive strategies. The popular amyloid cascade hypothesis suggests that AD is precipitated by the dysregulated metabolism of the amyloid precursor protein resulting in the accumulation of the beta-amyloid peptide (Aβ) in the brain. This hypothesis is the basis for most experimental treatments currently in clinical trials. However, not all evidence supports a precipitating role of abnormal Aβ accumulation in the common forms of AD.

The completion of the human genome project in 2001 has ushered in a dramatic increase in the technologies available to biologists and these have been applied to understanding the pathogenesis of complex disorders, like AD. Genome-wide association and expression (transcriptomic) studies are now commonplace creating a more immediate and dynamic, but invariably more complex, research environment.

Transcriptomic studies in particular have the potential to greatly influence our understanding of complex diseases like AD, but to date they have provided quite discordant results. There are a number of potential reasons for this including our incomplete understanding of the complex nature of the human brain at the molecular level. It is now known that the evolutionary advances in our cognitive capacity have largely originated at the level of transcription with substantial increases in both coding and non-coding RNA variants. Until recently transcriptomic platforms only assayed a proportion of the coding RNA species, but with the introduction of next generation sequencing the whole transcriptome, both coding and non-coding, can now be investigated.

In this chapter we undertake a review of the clinical and pathological characteristics of AD before considering the impact of new technologies on AD research and their future role in finding a cure for this important disease.

2. Alzheimer's disease

2.1 Overview

The term dementia encapsulates a number of separate diseases that clinically manifest as a progressive decline in cognitive function. Alzheimer's disease (AD) is the most common form of dementia accounting for approximately 60% of all cases. It is estimated that the current 36 million sufferers worldwide will increase to 115 million by 2050 (Alzheimer's Disease International, 2009). In countries like Australia, dementia will become the number one health cost by the middle of this century exceeding both heart disease and cancer (Access Economics, 2009). These surprising predictions result from the additive effects of an ageing world population, the duration of the disease and the extended period where the patient is totally dependent on caregivers. The societal trend in Western countries towards professional aged care services accounts for the majority of the current and predicted costs.

More importantly, AD is a cruel disease that robs patients of their sense of self and places considerable physical and psychological strain on caregivers, who are often isolated from society themselves. As the currently available treatments only provide transient improvement, an AD epidemic will only be halted with the development of new therapies. It has been predicted that a novel therapeutic agent that delays disease onset and progression by just one year would result in nine million fewer cases by 2050 (Brookmeyer *et al.*, 2007).

The other approach to reducing the worldwide burden of AD is prevention. This would be based on either avoiding identified risk factors or augmenting the impact of protective factors. Unfortunately finding such factors through epidemiological studies has proved extremely difficult to date.

The completion of the human genome project in 2001 ushered in a dramatic increase in the technologies that have subsequently been applied to understanding the pathogenesis of complex disorders, like AD. Genome-wide association and expression studies are now commonplace in AD research creating a more immediate and dynamic research environment. The application of these technologies to clinical, pathological and epidemiological studies of AD is starting to bear fruit although effective treatments are not necessarily close at hand.

2.2 The history of AD

Emil Kraepelin first used the name Alzheimer's disease in 1910 in recognition of Alois Alzheimer (1864-1915), the German psychiatrist and neuropathologist, who had originally described the clinical and pathological features of the disease. Kraepelin was keen to make the distinction between the presenile form of dementia (described by Alzheimer) and the more common senile variant.

Alzheimer's findings were originally published in 1907, in the form of a conference abstract where he described a delusional woman (Auguste D) who had slowly lost her cognitive function and died at 55 years of age (Alzheimer, 1907). According to a later translation of the original manuscript Auguste D suffered from reduced comprehension and memory as well as psychotic symptoms including paranoia that resulted in her psychosocial impairment (Maurer *et al.*, 1997). The first formal description of Auguste D was by Perusini in 1909 in a

textbook edited by Nissl and Alzheimer (Perusini, 1909). Alzheimer himself only published a detailed clinical and pathological report in 1911 along with his findings from a second case (Johan F) (Alzheimer, 1911). Interestingly Alzheimer described Auguste D as having both the pathologies that we now know as plaques and tangles in her cerebral cortex but Johan F as having only plaques. Graeber and colleagues re-examined Alzheimer's original sections 90 years later and confirmed the original histopathological findings for Johann F (Graeber *et al.*, 1997) and Auguste D (Graeber *et al.*, 1998). In accordance with Kraepelin's original classification the term Alzheimer's disease or Alzheimer's presenile dementia continued to be exclusively used for a rare disease of mid-life until the 1970's when neuropathologists realised that the more common senile dementia and the type described by Alzheimer were indistinguishable (Terry and Katzman, 1983).

2.3 Prevalence and cost

The mean age at onset of AD is around 75 years of age and the overall prevalence is about 1% in most developed nations. If AD prevalence is divided into different age bands then we see a rapid increase from 1% in the 60-64 year age group to greater than 25% in individuals over 85 years of age (Jorm and Jolley, 1998).

A comprehensive estimate of worldwide prevalence was recently reported with age- and gender-specific prevalence and projections out to 2050 (Alzheimer's Disease International, 2009). This suggested that the current worldwide burden of 35.6 million dementia sufferers would nearly double every 20 years, to 65.7 million in 2030, and 115.4 million in 2050. The authors attributed this increase to population growth and demographic ageing.

Alzheimer's Disease International (ADI) has addressed the current cost of dementia worldwide and predicted its future economic impact (Alzheimer's Disease International, 2010). They suggested that the currents cost of US$604 billion would increase by 85% to US$1000 billion by 2030. They noted considerable disparity between high-income and low-income countries largely due to formal care facilities in the former, rather than direct health costs. Direct health costs amounted to only 16% of the average $32,000 per annum costs in the USA. ADI considered that stigmatisation played a role here where cognitive decline was often the precipitant for institutionalisation as opposed to individuals with severe physical disabilities who are often supported at home by community services.

In terms of care-giving an important indirect effect of AD and dementia that may often be missed in such reports is the physical and psychological toll on the caregivers themselves. It is estimated that a person with dementia requires more than 7 hours of close supervision per day (Wimo *et al.*, 2007). Caregivers are commonly spouses or first-degree relatives, and for long periods of time will be as socially isolated as the patients themselves. They are estimated to experience between a three- and 40-fold increase in their risk of major depression (Cuijpers, 2005). Caregivers who choose to support a dementia patient at home also are likely to suffer a huge financial burden, through lost of income, disability-friendly renovations and the hiring of respite care. These costs are only partially off set by government allowances or pensions in most cases.

2.4 Clinical findings

The term dementia refers to the main clinical manifestation of AD but also occurs with other diseases including frontotemporal dementia (FTD), vascular dementia (VaD) and dementia

with Lewy bodies (DLB). Dementia is a progressive decline in cognition that impairs social and/or occupational function. The deficits in a particular individual will reflect the regions in which neuronal loss occurs.

AD sufferers will usually present to a general physician because of their own concern about deteriorating cognition or the concerns of an informant (often their spouse or work colleague). A general physician may refer the patient to a specialist geriatrician or neurologist but many patients remain under the primary care of their general practitioners. The loss of short-term memory is often the earliest cognitive phenotype for AD (Dubois and Albert, 2004).

2.5 Diagnosis

The criteria for a clinical diagnosis of AD were established in 1984 and have remained unchanged for the past 27 years (McKhann *et al.,* 1984). The diagnosis of AD is currently based on clinical examination and neuropsychological testing although a definitive diagnosis can only made by postmortem examination. However, the latter is uncommon outside a research setting. A diagnosis of 'Probable AD' is made when there is worsening memory function, particularly episodic memory, accompanied by deficits in two or more other cognitive domains such as executive function, attention, language or visuospatial skills.

A diagnosis of AD would be supported by a positive family history, a normal electroencephalogram, normal cerebrospinal fluid (CSF) analysis and atrophy on computer-assisted tomography (CAT) imaging (the only neuroimaging modality available in 1984). An alternative diagnosis of 'possible AD' was suggested when there was a general dementia syndrome but with variations in onset and clinical course (McKhann *et al.,* 1984). Exclusion criteria for AD included the rapid onset of dementia, an early prominence of gait abnormalities or a focal neurological deficit.

In 2011, these criteria were updated, prompted by some inconsistent clinical-pathological correlations and a need to incorporate the likely prodromal periods of AD and biomarkers (biological markers) (Jack et al., 2011). The new criteria propose a continuum of impairment but also delineate three stages along this spectrum: preclinical AD, mild cognitive impairment (MCI) and clinical AD. The basis for these stages is that the pathophysiological process of AD can be reliably represented by biomarkers. The existing 'probable' and 'possible' categories are now expanded to (1) Probable AD dementia, (2) Possible AD dementia, and (3) Dementia with evidence of the AD pathophysiological process. An example of the latter would be an individual with a less typical clinical presentation but with AD-like biomarkers on their neuroimaging or CSF analysis (McKhann *et al.,* 2011). However, biomarkers continue to only support a clinical diagnosis of AD while definitive diagnosis still requires a postmortem examination.

The differentiation of AD from MCI rests on the determination of whether or not there is significant interference in the ability to function at work or in usual daily activities (Albert *et al.,* 2011). MCI is not a definite stage on the progression to AD because some individuals will never develop dementia or will develop other forms such as VaD. However, pathological studies suggest that the majority of individuals with MCI have typical AD pathology, but just in a lesser amount to those who become clinically demented (Haroutunian *et al.,* 2009).

The definition of preclinical AD remains a research-only paradigm based on the promise that biomarkers faithfully reflect the underlying pathophysiological processes (Sperling *et al.*, 2011). As will be discussed below this preclinical diagnostic paradigm is firmly based on one, albeit prominent, working hypothesis for AD pathogenesis.

2.6 Clinical heterogeneity

The review of the clinical diagnostic criteria was prompted in part by increasing reports of variations in the classic presentation of AD. It has even been suggested that the term Alzheimer's syndrome is more appropriate than AD (Zellner *et al.*, 2009). In addition to the classic amnestic presentation of AD described above, a "visual variant" of AD, often called posterior cortical atrophy, a "language variant" called logopenic/phonological aphasia (Gorno-Tempini *et al.*, 2011) and a frontal/behavioural variant are occasionally observed (Johnson *et al.*, 1999). The latter two are also considered sub-categories of frontotemporal dementia hinting that there might be considerable overlap in clinicopathological correlates between the current clinical dementia categories. This is supported by a recent study that found AD to be the primary pathological diagnosis in a range of focal cortical syndromes including 50% of corticobasal disease and 44% progressive non-fluent aphasia, another language variant of frontotemoporal dementia (Alladi *et al.*, 2007). This clinical or phenotypic heterogeneity in AD suggests variation in both aetiology and pathogenesis and the potential ramifications of this will be discussed in greater detail below.

2.7 Current treatment options

There are two classes of drugs that are currently used to treat moderate to severe cases of AD. They are the acetylcholinesterases inhibitors (ChEIs) and glutaminergic receptor antagonists. The cholinergic (acetylcholine producing) neurons of the basal forebrain nuclei such as the nucleus basalis of Meynert are among the earliest lost in AD (Cullen and Halliday, 1998). These neurons synapse in the prefrontal cortex and the hippocampus where they release acetylcholine to modulate circuits involved in learning and memory (Gold, 2003).

Acetylcholinesterases are enzymes responsible for the rapid breakdown of acetylcholine in the synaptic cleft. ChEIs are postulated to maximise the residual acetylcholine modulating these memory circuits in the AD brain and there are three types in current use donepezil, rivastigmine and galantamine. Meta-analyses of randomised control trials for the three ChEIs suggest that all show moderate improvements in cognitive function over placebo but the incidence of adverse events is lowest for donepezil (Birks, 2006; Hansen *et al.*, 2008)

The other drug in common use is called memantine, a low affinity glutamate NMDA receptor antagonist. The postulated mechanism of action for memantine is the prevention of glutamate-mediated excitatory neurotoxicity (McShane *et al.*, 2006). As with the ChEIs, meta-analyses report a significant but marginal improvement (Raina *et al.*, 2008) with particular attenuation of behavioural symptoms (Gauthier *et al.*, 2008). Memantine is less efficacious in mild to moderate compared to severe AD (MMSE >15) (McShane *et al.*, 2006; Schneider *et al.*, 2011).

Unfortunately there is no evidence that either of these treatments significantly alters disease progression (Golde *et al.*, 2011). Hence there is enormous research effort aimed at finding alternative treatment strategies, some of which have already reached Phase II and III human clinical trials. These potential treatments are discussed below.

2.8 Neuropathology

As discussed in the section above, a definitive diagnosis of AD can only be made at postmortem examination. Unfortunately the number of autopsies has declined markedly in the last three decades depriving clinicians and researchers alike of an important source of medical knowledge. Neuropathological examinations are now largely restricted to a research environment with the potential issue that clinically confirmed cases are not be representative of the AD spectrum in the greater population.

As with the clinical diagnosis, there are established criteria for the pathological diagnosis of AD (Hyman and Trojanowski, 1997) although these too are currently being revised. Interestingly, although postmortem examination is required for a definitive diagnosis of AD the pathological criteria again, only suggests that AD is 'probable' or 'possible'. In order to understand how these criteria have been derived it is useful to give a brief overview of the characteristic or pathognomonic aspects of AD.

2.8.1 Gross pathology

The gross pathology of the end-stage AD brain is characterised by widespread atrophy due to the extensive neuronal loss. There is narrowing of gyri and widening of sulci with atrophy of the temporal cortices and a disproportionate atrophy of the entorhinal cortex, amygdala and hippocampus in particular. The remaining cortical regions are generally atrophic, but to a lesser extent than the temporal lobe, although there are also regions seemingly spared from neuronal loss such as the inferior frontal cortex (Halliday *et al.*, 2003) (Fig. 1(a-b)).

2.8.2 Plaques and tangles

The neuropathology of AD is characterised by two pathognomonic entities; the intraneuronal neurofibrillary tangle (NFT) and the extracellular plaque. NFTs are chiefly composed of hyperphosphorylated fibrillar forms of a protein called microtubule associated protein tau (MAPT or tau), while plaques are predominantly composed of fibrils of peptides collectively termed beta-amyloid (Aβ) (Fig. 1 (c-d)).

For most individuals, there is a good correlation between spread of NFT pathology from the medial temporal lobe to the association and finally primary cortices, and the probability of dementia (Newell *et al.*, 1999). In comparison the regional distribution of plaques tends to be highly variable between patients (Braak and Braak, 1991). Similarly plaques vary more in their cortical laminar pattern whereas NFTs are found mainly in layers III and V coinciding with the corticocortical projection (pyramidal) neurons (Lewis *et al.*, 1987). Plaques also vary in their structure and are referred to as 'diffuse' (lacking associated inflammatory cells and dystrophic neurites) and 'cored' or 'neuritic' plaques. It is generally considered that diffuse plaques eventually become cored but an alternative explanation suggests that the amyloid of diffuse and cored plaques are derived from blood vesssels and neurons respectively (D'Andrea and Nagele, 2010).

Extracellular Aβ deposition is considered to be the most likely precipitating event in the disease (Hardy and Selkoe, 2002). In comparison, NFT formation is a subsequent but necessary step in neurodegeneration of AD with intracellular tangles becoming 'ghost tangles' when the neuron eventually succumbs and the insoluble tau protein is left behind in the parenchyma. Presumably the brain macrophages find these extracellular tangles too difficult to phagocytose or they are relatively inert.
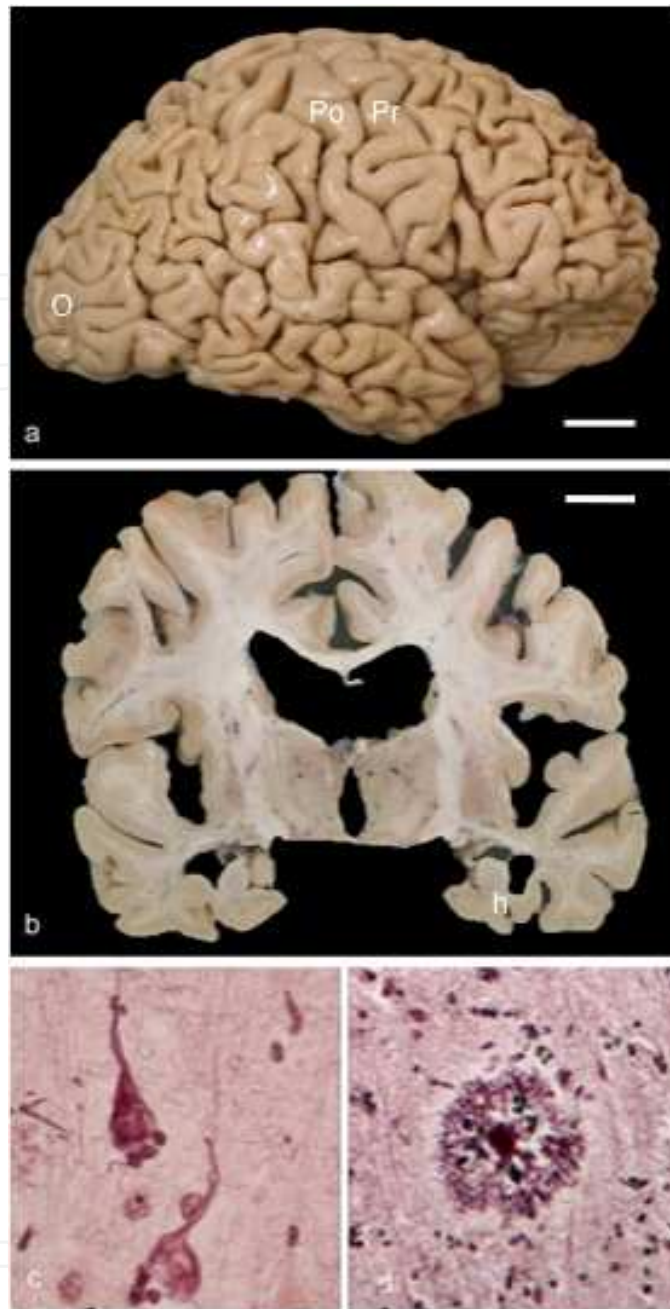
Fig. 1. The neuropathological features of Alzheimer's disease. A series of macro-and microscopic images show the pathological features of Alzheimer's disease (a) A lateral view of the right cerebral hemisphere shows extensive atrophy of the temporal and frontal lobes. There are also regions spared in this case including the precentral (Pr) and and postcentral (Po) gyri and the occipital pole (O), size bar = 2cm (b) A coronal view at the level of the lateral geniculate body demonstrates the severe atrophy in the temporal lobe including the hippocampus (h), along with enlargement of the Sylvian fissure, and third and lateral ventricles, size bar = 2cm (c) A modified Bielschowsky's silver stain shows neurofibrillary tangles in two cortical pyramidal neurons, magnification = 400x and (d) a cored plaque, with its dense amyloid core surrounded by diffuse amyloid, dystrophic neurites and cellular debris, magnification = 200x.

2.8.3 Neuronal loss

The hippocampal CA1 (1st cornus ammonis or Ammon's horn) region experiences the greatest loss of neurons in AD of approximately 70%. The CA1 is anatomically and functional connected to the entorhinal cortex where the earliest development of NFTs and neuronal loss in AD is thought to occur. The locus coeruleus (noradrenalin production), nucleus basalis of Meynert and the Raphe nuclei can also experience losses of greater than 50% as does the majority of the temporal lobe (Kril and Halliday, 2001). The loss of neurons in the nucleus basalis of Meynert is similar to the number of ghost tangles but in the hippocampus (Kril *et al.*, 2002) and entorhinal cortex (Gomez-Isla *et al.*, 1996) neuron loss actually exceeds the number of ghost tangle suggesting other neurodegenerative mechanisms are at work. The spread of pathology and associated neuronal loss in AD dictates the symptomology. The reader will note the early involvement of the hippocampus, a key region in both memory generation and consolidation. Interestingly the eventual spread of AD pathology mirrors the anatomical boundaries of the default network – the "brain system active when individuals are not focused on their external environment" (Buckner *et al.,* 2008). The default network is involves with the process of internal mentation or self-relevant mental simulation. This peculiarly human activity could explain the regional selectivity of neuronal loss in AD while deficits in the network are certainly consistent with the loss of self-awareness in moderate to severe stages of the disease (Buckner *et al.*, 2008).

2.8.4 Chronic inflammation

AD is also characterised by a chronic inflammatory process that is commonly called reactive gliosis. Markers of inflammation such as MHC II expression are higher in demented patients than those in nondemented individuals with AD pathology and may be better correlated with synaptic dysfunction than either plaques or NFTs (Lue *et al.*, 1996). Essentially when we refer to reactive gliosis we mean reactive microgliosis, a hyperplastic and hypertrophic response of the resident macrophages in the brain. There is also astrocyte pathology but this remains less well defined in AD (Beach and McGeer, 1988). The increased expression of the cytosketal protein glial fibrillary acidic protein (GFAP) that characterised 'reactive' astrocytes has been described in AD brains although this was not correlated with either plaques or NFTs (Simpson *et al.*). Microglia are originally derived from the haemopoietic system and migrate to the brain during the early embryological period. Reactive microglia in AD are closely associated with plaques and studies with immunomodulatory therapies suggest that they are relatively effective, at least early in the disease process, at phagocytosing Aβ plaques (Perlmutter *et al.,* 1990; Edison *et al.,* 2008). However neuroinflammation appears to exacerbate AD pathogenesis (Krause and Muller, 2010) and anti-inflammatory medication use is associated with a reduced risk of AD (Vlad *et al.*, 2008). This apparent paradox might be explained by microglia initially serving a protective role but eventually becoming overstimulated and producing excessive reactive oxygen species that lead to neurotoxicity (Innamorato *et al.,* 2009). Nevertheless anti-inflammatory medications do not reduce the progression of AD pathology (Halliday *et al.*, 2000). As microglia are now known to have physiological functions in the brain such the maintenance of synaptic plasticity and it has been suggested that they may be more 'victim' than 'villain' in AD (Graeber and Streit, 2010).

2.8.5 Diagnostic criteria

The current pathological criteria are based on the quantity of both plaques and NFTs (Hyman and Trojanowski, 1997) and incorporate a staging scheme for the neuropathological progression of NFTs ('Braak staging scheme') (Braak and Braak, 1991). The likelihood of AD is high if there are frequent neocortical plaques and NFTs consistent with Braak stage V/VI; is intermediate if plaques are moderate with Braak stage III/VI and low if neocortical plaques are sparse with Braak stage is I/II.

2.9 The ageing brain

One of the reasons that the AD neuropathological diagnostic criteria are a probability scale is the occurrence of both plaques and NFTs, either independently or together, in a proportion of aged subjects without dementia. Diffuse plaques are seen in as many as 30% of all brains examined (Braak and Braak, 1997; Davis *et al.*, 1999). A similar percentage of cognitively normal individuals have tau-positive neuritic pathology (Knopman *et al.*, 2003) although tau pathology confined to the hippocampal formation appears to be seen in most, if not all, aged brains (Troncoso *et al.*, 1996). As discussed above the quantity of AD pathology tends to correlate relatively closely to the level of dementia and many researchers consider that the presence of mild pathology is representative of preclinical AD (Price *et al.*, 2009). Dementia then occurs when the quantity of AD pathology crosses a particular threshold. Two issues with this idea are that some individuals with Aβ will not develop dementia prior to death (incidental AD pathology) and these Aβ clinicopathological correlations tend to breakdown in the oldest old (>90 years of age) (Kril, 2009).

As will be discussed further below, the common occurrence of incidental AD and AD-type pathology in nondemented controls could also be a factor in the small effect sizes and lack of reproducibility seen in AD association studies to date (Sutherland *et al.*, 2011b).

3. The genomic era and its new technologies

The last decade has seen an incredible advance in the biologist's armamentarium for investigating complex diseases. This biological revolution has its roots in the development of molecular biology techniques such as gene cloning using restriction enzymes (Nathans and Smith, 1975), DNA hybridisation (Southern, 1975), Sanger sequencing (Sanger *et al.*, 1977) and the polymerase chain reaction (PCR) (Saiki *et al.*, 1985; Mullis and Faloona, 1987). However, it was human genome project that provided the real impetus.

3.1 The human genome project

The human genome project began in 1989 and was initially headed by the co-founder of DNA's structure and Nobel laureate, James D Watson. He was succeeded by Francis Collins who headed the public sequencing effort while a private consortium (Celera) also undertook the challenge of sequencing the entire (haploid) human genome, an estimated three billion base pairs. The twin consortia announced the completion of the draft human genome in late 2000 (public (Lander *et al.*, 2001) and Celera (Venter *et al.*, 2001)) although a so-called completed version was only announced in 2003. The templates for these human genomes were pooled DNA samples while the entire genome of a single individual was only

published in 2007 (Levy *et al.*, 2007). However it is currently estimated that actually only 93% of the human genome has been sequenced with repeat regions within telomeres and centromeres remaining outstanding. The public contribution to the first human genome is estimated to have cost US$3 billion.

It was predicted prior to and during the project that the human genome would contain between 40,000 and 100,000 protein coding genes and this number would greatly exceed other mammals, explaining, in particular, our cognitive uniqueness. However, it now appears that we have only about 20,000 to 25,000 protein-coding genes (1-2% of the entire genome), a figure that is, not only similar to other mammals, but dwarfed by some plant species (International Human Genome Sequencing Consortium, 2004).

In disease research we are particularly interested in the genetic variation between affected and unaffected individuals. As the sequencing of whole genomes for all the individuals in a research cohort remains prohibitively expensive, researchers have had use to methods that can approximate the total genetic variation.

3.2 Genetic variation

In monogenic forms of diseases such as AD, single, rare genetic polymorphisms that we commonly call mutations cause the disease. These mutations generally segregate in families or very rarely originate *de novo* in the germ line of our patient of interest (proband). The former have been, and will continue to be for the next few years at least, discovered by gene linkage studies in multi-generational families.

In contrast most AD cases are regarded as sporadic with no familial or geographic clustering. They are also called idiopathic (literally unknown pathogenesis). However despite lacking a Mendelian pattern of inheritance we do know that genetics contributes to the susceptibility of sporadic AD. What we do not know for any particular sufferer is the magnitude of that genetic contribution or the number of genes involved. The 'common variant' hypothesis for complex diseases states that a variable number of commonly occurring gene variants combine to make up the genetic component of disease causation. A common variant or polymorphism is defined as one where the minor allele frequency is greater than 1% in the population.

The most numerous genetic variants are single nucleotide polymorphisms (SNPs) and there are thought to be around 30 million SNPs in total meaning that two unrelated persons, chosen at random, will differ at about 1 in every 1,200 to 1,500 bases. In 2002 the National Institutes of Health started a $138 million project called the HapMap Project to catalogue the common SNPs in European, East Asian (Han Chinese and Japanese and African (Yoruba)) populations. By 2010 HapMap had released details of SNPs and inferred copy number polymorphisms in 1300 individuals from these four ethnic groups (Altshuler *et al.*, 2010). As will be discussed below, this detailed data on human genetic variation combined with gene chip technology allows the simultaneous testing of more than 2 million SNPs in what are called genome wide-association studies (GWAs).

The remaining 'uncatalogued' common variants will consist of rarer SNPs and smaller copy number variations (<500 base pairs) (Conrad *et al.*, 2010). The '1000 human genome project' aims to use next generation sequencing (NGS) to bridge this gap (The 1000 Genomes Project

Consortium, 2010). NGS has many current and potential applications in studying complex diseases and will be a recurring theme for the remainder of this chapter.

3.3 Microarrays

Microarrays (a generic term for a gene chip) are utilised for GWAs but they are more commonly known as a platform in genome-wide expression or transcriptomic studies. Microrrays were initially manufactured by printing the cDNAs of interest onto a glass microscope slide but now short oligonucleotides (probes) complementary to known exonic regions are directly synthesised onto a glass microscope slide or microscopic beads. Probes are typically designed to complement (bind to) the 3′ untranslated regions of transcripts although newer generation arrays have multiple probes across whole genes. The detection of signal with the microarray platform relies on probe-hybridisation and non-specific binding can potentially affect the relative detection of lowly expressed transcripts. Modern microarrays have around 50,000 probes representing the approximately 20,000 known protein coding genes, meaning there is redundancy for some genes. The accompanying software will usually report only the maximally expressed or maximally differentiated probe for each gene. The ability to make 20,000 comparisons in a single experiment is an extremely powerful and seductive tool that importantly makes no *a priori* hypotheses to the importance of any one gene. However, this number of comparisons always exceeds the number of individuals being sampled creating statistical dilemmas for the confident detection of real differences. This 'curse of multidimensionality' similarly affects the analysis of GWAs or any –omic platform (Somorjai *et al.*, 2003).

3.4 Next generation sequencing

The sequencing of the human genome project was carried out using a technique called dye terminator chemistry developed by Fred Sanger 30 years before (Sanger *et al.*, 1977). This technique relies on the amplification of the DNA sequence with incorporation of terminating dideoxynucleotides and the subsequent arrangement of all the fragments (now by software) to derive the final sequence. Although capable of long accurate reads (up to 1500 bases) and eventually automated with capillary technology the technique was, by today's standards, very slow.

The term next generation sequencing (NGS) generically refers to platforms that allow the sequencing of several hundred thousand templates simultaneously (Margulies et al., 2005). For most NGS platforms the multiple templates are sequentially exposed to a known nucleotide whose attachment to its complementary base generates ATP, which is subsequently used to produce a luminescent signal. The signals across the array are captured as an image, before the excess nucleotides are washed away and the process repeated. The actual cycle number or read length is platform-dependent, but will vary from 75 to about 400 bases. This in-parallel sequencing is obviously much quicker than the single-sample Sanger method and now allows relatively small laboratories with a single instrument to perform whole genome sequencing that was, until very recently, the domain of dedicated sequencing centres. There are now also third generation sequencing systems based on single-molecule analysis. These promise to be quicker and cheaper than NGS platforms and more accurate due to longer read lengths although they may still not quite achieve the lengths seen with Sanger sequencing.

One of the major applications of NGS is full-length mRNA sequencing called RNA-Seq. Unlike microarrays RNA-Seq provides a digital readout of all transcripts including those that are lowly expressed and it does not rely on prior knowledge of the genome for probe design. Most current preparatory methods for RNA-Seq continue to use a PolyA fraction (mRNA) but total RNA methods, which require the deletion of the abundant architectural RNA species (ribosomal and transfer RNA), are improving. The latter allows the full repertoire of both coding and non-coding RNA to be quantified.

3.5 Proteomics

The term proteome describes the full complement of proteins, including post–translational variants, produced in a particular cell or tissue (Wilkins *et al.*, 1996). For many biologists, proteins remain the key functional entities that can only be approximated by transcriptomic analyses. Certainly there is not necessarily a linear relationship between mRNA and proteins levels. In a generic proteomic analysis, the lysate would be separated by two-dimensional gel electrophoresis and the 'spots' of interest excised, digested, and the resultant peptide fragments subjected to mass spectroscopy (MS). Individual spectra are compared to databases to derive peptide identity and by computation, the likely parent protein. This process is also referred to peptide mass fingerprinting. It is now more common to use tandem MS where a specific peptide is further fragmented and fragments subjected to MS (peptide fragmentation fingerprinting). Proteomics can be made semi-quantitative by spiking in stable isotopes as reporter ions allowing the relative abundance of the peptides in the overall spectra to be calculated.

4. Pathogenesis

Pathogenesis is defined as the series of events and mechanisms that result in the clinical manifestation of a disease. It encompasses the aetiological agent, its entry into the host and the subsequent host responses to that agent over time. In general the greater the 'dose' of an aetiological agent, the more rapid will be the disease onset and severity. Host resistance or compensatory mechanisms will act to maintain homeostasis and so delay the disease onset and its impact, but an over exuberant or aberrant host response could also be a major factor in the clinical manifestation of the disease itself. Neurodegenerative diseases tend to be late-onset, around 75 years in the case of AD, with preclinical or prodromal periods of extended, but unknown, lengths. This type of disease history presents a number of pathogenic possibilities. In one scenario the aetiological agent(s) are extremely subtle and require a long time period to overcome host resistance. Alternatively host resistance may be entirely capable of dealing with the neurodegenerative agent until it fails due to old age. In the latter case a much shorter prodromal period would be expected. A variation on the latter theme is that rather than a foreign agent inducing the pathogenic process, it is a physiological process that goes awry because suitable checks and balances fail with advancing age. If the latter proved to be the case with AD then treatment strategies might be plagued by unwanted side effects.

In the introduction we discussed that there are two major forms of AD, monogenic and sporadic. These types of AD are sometimes referred to early-onset (EOAD) and late onset

(LOAD), with 60 years of age often given as the arbitrary cut off. With reference to monogenic forms of AD we might think of mutations as an increased 'dose' (severity) of an aetiological agent and monogenic AD can present as young as 20 years of age. In fact, very rarely, AD-causing mutations are actually gene multiplications with direct gene dosage effects.

Sporadic forms of a disease are defined by having no familial or geographic clustering but this term is slightly misleading because there is certainly a genetic component in these common forms of AD. Epidemiological studies suggest that AD sufferers have a 2.5 fold greater likelihood of family history of the disease (Sutherland *et al.*, 2011b). In our 'dose' analogy above the late-onset nature of these common forms of AD would be consistent with common genetic variants, conferring only slight alterations to protein function or expression, being the causative agent. We also generally assume that the genetic component will be multifaceted with both additive and interactive relationships with environmental exposures. The latter refers to a scenario where a potential genetic risk factor only modifies disease risk if that individual has been exposed to a certain environmental stress. We will return to the discussion of AD genetics shortly but it is useful at this stage to introduce the amyloid precursor protein (APP) and its metabolite Aβ.

4.1 APP metabolism

The Aβ peptide was initially purified from amyloid-containing AD brain tissue (Glenner and Wong, 1984) and the cored plaques of AD and Down syndrome patients (Masters *et al.*, 1985). The parent protein, APP, was then isolated from a human brain cDNA library (Kang *et al.*, 1987). The predicted 695 amino acid long protein with a single transmembrane domain was described as being similar to the prion protein, a hypothesised neuronal surface receptor. There are three major APP isoforms, 695, 751 and 770 amino acids in length with APP_{695} being the most common in neural tissue (Yoshikai *et al.*, 1990).

Following translation the APP protein is retained in the secretory pathway, and is transported to the cell membrane. The protein is subsequently degraded via proteolytic cleavage by either α-, and γ-secretases or alternatively β- and γ-secretases at the cell membrane or following the endocytosis of APP as part of normal membrane turnover (LaFerla *et al.*, 2007). In addition a variable fraction of APP undergoes post-translational proteolytic cleavage within the secretory pathway. The γ-secretase is a multi-unit enzyme, that includes either the presenilin 1 (PS1) or presenilin 2 (PS2) proteins and it cleaves APP (and other proteins such as Notch) within its transmembrane domain. α-secretase activity is carried out by a family of proteins (including ADAM 9) and this cleavage results in the secretion of an extracellular fragment of APP (sAPP-α) and the retention of a C-terminal fragment (CTF) of 83 amino acids (Fig. 2). sAPPα appears to act as a neuroprotectant, and has neurotrophic effects on synaptic plasticity (Postina, 2008). Alternatively, and mutually exclusive to α-secretase cleavage, the β-secretase enzyme (BACE1, also called Asp2, memapsin 2, is the major form in the brain) cleaves APP at variable sites about 16 amino acids proximal to the α-secretase site (Vassar *et al.*, 2009). The combination of β-secretase and γ-secretase cleavages releases a variety of peptides that are collectively called Aβ (1,2,3 to 39-43) (Fig. 2).
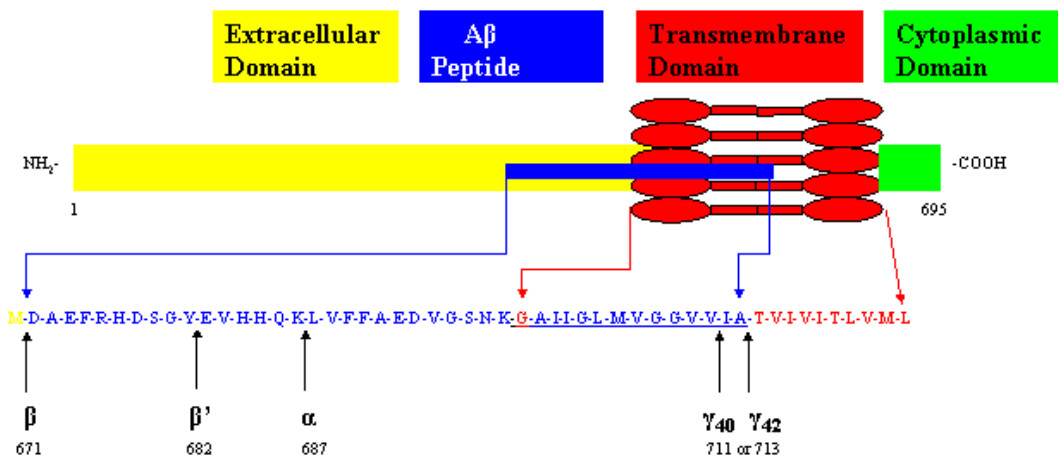
Fig. 2. The structure of the amyloid precursor protein (APP$_{770}$). This is a schematic diagram of the longest APP isoform (APP$_{770}$). The α-, β-and γ-secretase sites are shown. APP undergoes physiological cleavage through two alternative pathways; α-secretase or β-secretase. The latter pathway involves the cleavage of APP at β and γ sites and results in the production of various Aβ peptides. Cleavage at the α site prohibits the formation of the Aβ peptides. The hydrophobic 29-40(2) segment of the Aβ peptide is part of the transmembrane domain of APP and is likely to confer both aggregative and membrane–binding behaviour to these peptides (Sutherland, 2003, PhD thesis).

The β-secretase pathway is also called the amyloidogenic pathway because the major products Aβ$_{1-40}$ and Aβ$_{1-42}$ will rapidly aggregate with themselves *in vitro* and *in vivo* with Aβ$_{1-42}$ in particular capable of rapid self-assembly. Aβ$_{1-42}$ is known to be neurotoxic *in vitro* (Lorenzo and Yankner, 1994) and *in vivo* (see animal models below) and thought to be central to AD pathogenesis.

Amyloid structures are composed of pairs of anti-parallel β -strands or β-pleated sheets of Aβ. It was these fibrillar forms of Aβ that were originally presumed to be pathogenic although oligomers are now generally regarded as the pathogenic form of Aβ (Walsh *et al.*, 2002). Interestingly Aβ is produced in proportion to synaptic activity and has the opposite effect to sAPPα on synaptic connections (Selkoe, 2002). This could suggest an useful antagonistic action although Aβ is still generally regarded purely as a degradative product. Aβ is normally removed from the brain by the perivascular drainage of interstitial fluid to the cervical lymphatics (Weller *et al.*, 2008) but is also actively secreted into the CSF.

4.2 Monogenic forms

The first AD family presenting with an apparent Mendelian pattern of inheritance was described in 1932 (Schottky, 1932). In more modern times a family was reported with 51 affected persons in 8 generations (Nee *et al.*, 1983). Down syndrome individuals who are trisomic for chromosome 21 (C21) and have 3 copies of the APP gene also develop Aβ pathology (Heston, 1977). A gene dosage model was proposed for Down syndrome patients as an explanation for early presentation with AD-like pathological changes (Tanzi, 1989).

Early linkage studies of multiple large kindreds mapped a common genetic defect to the *APP* region of C21 (St George-Hyslop, 1987 #8988) although *APP* did not initially appear to be involved (Tanzi *et al.*, 1987a; Tanzi *et al.*, 1987b). Missense mutations in *APP* was eventually described in 1991(Goate *et al.*, 1991) while the predicted duplications in the APP gene, given the gene dosage hypothesis for Down syndrome, were only discovered later (Rovelet-Lecrux *et al.*, 2006). These cases also showed cerebral amyloid angiopathy, a disease entity that can occur independently of AD but is present in 80% of sporadic AD cases (Ellis *et al.*, 1996). APP mutations themselves are very rare and it is the PSEN1 gene (encoding presenilin 1) where the majority of AD mutations have been detected (Sherrington, 1995 #674). Mutations in the PSEN2 gene were also found in 1995 (Levy-Lahad *et al.*, 1995) but these are also relatively uncommon (Bekris *et al.*, 2010). In comparison to *PSEN1* mutation carriers the PSEN 2 cases had a more variable phenotype, a later onset of disease and a reduced penetrance, but the ratio of Aβ $_{1-42}$:Aβ $_{1-40}$ is increased in all monogenic AD brains.

One of the PSEN1 families (N141I mutation) was descended from Volga Germans who had immigrated to the USA. It was later suggested that these individuals were likely to originate from the same region of Germany as Auguste D (Yu, 2010 #8999) and perhaps she had the same mutation.

4.3 Amyloid cascade hypothesis

There is almost irrefutable proof that monogenic forms of AD result from the aberrant production or metabolism of the Aβ peptides and these rare forms are, apart from the earlier age at onset, phenotypically very similar to the sporadic forms (Shepherd *et al.*, 2009). This similarity was the major driver for Hardy and Higgins to propose the amyloid cascade hypothesis for the pathogenesis of sporadic AD in 1992. This hypothesis suggested that the neurotoxic Aβ sets up a cascade of events in adjacent neurons resulting in NFT formation and neuronal death. How Aβ actually precipitates these events was not articulated but most interpreted the hypothesis as suggesting that it was Aβ fibrils in the form of plaques that were the neurotoxic entity. Later amendments to the hypothesis suggested that it was more likely to be Aβ$_{1-42}$ oligomers rather than fibrils but whether these interact directly with the neuronal cell membrane or via receptors was still unknown (Hardy and Selkoe, 2002). The cascade hypothesis remains the most popular working hypothesis for AD pathogenesis and is the underlying basis for proposed preclinical diagnostic criteria and the vast majority of treatments under development (Sperling *et al.*, 2011). Nevertheless, it is not quite unanimously accepted. Distracters, in particular, point to the fact that NFTs rather than amyloid are initially deposited in the memory-associated hippocampus and that the regional progression of NFTs is a better correlate of disease symptoms and severity (Braak and Braak, 1991). Furthermore the latest GWAs also hint at Aβ-independent mechanisms for sporadic forms of the disease (discussed below).

The monogenic forms of AD have also allowed the production of animal models, the main research workhorse towards understanding pathogenesis. A brief synopsis of the mice models is given below but the area has been extensively reviewed by Gotz and Ittner (Gotz and Ittner, 2008) including a thorough consideration of how invertebrate models such the fruitfly (*Drosophila melogaster*) and the worm, *Caenorhabditis elegans*, have also impacted on AD research.

The transgenic expression of human mutant APP in mice (Games *et al.,* 1995) caused plaques and inflammatory responses but not tangles or any substantial neuronal loss. These mice were subsequently crossed with a variety of gene knockout strains including an apolipoprotein E (APOE) null mouse where a dramatic reduction in Aβ load was seen (Bales *et al.,* 1997). As we will discuss shortly, the possession of the ε4 variant of the APOE gene is a leading risk factor for sporadic AD. Holtzman and colleagues were then able to show in these mice that the additional expression of human APOE ε4 produced a 10-fold greater increase in Aβ deposits than APOE ε3 (Holtzman *et al.,* 2000). A double transgenic mouse was subsequently developed with both APP and PSEN1 mutant forms and this showed both an acceleration of Aβ deposition and an increased quantity of Aβ load over either parent mutant mouse (Holcomb *et al.,* 1998).

Aβ-orientated studies have dominated the AD field but there has always been a sub-community of researchers that have investigated the role of tau. The first tau transgenic mouse was produced in 1995 and showed that exogenous tau was redistributed away from the axon and hyperphosphorylated in a 'pre-tangle' state (Gotz *et al.,* 1995). Although no mutations in the tau gene (MAPT) have been found in AD patients, tau mutations have been described in a familial form of frontotemporal dementia proving that mutant tau is sufficient for neurodegeneration (Hutton *et al.,* 1998). The first mutant tau mouse strain was produced in 2000 (Lewis *et al.,* 2000) and then a double mutant APP/tau cross was produced in 2001 with the expectation that this would be an accurate phenocopy of AD (Lewis *et al.,* 2001). This double mutant showed the same Aβ pathology as the APP mutant mouse but greater NFT pathology than the parent mutant tau mouse strain and greater neuronal loss. This suggested a potential interaction between Aβ and tau, a possibility strengthened by the demonstration that the intracerebral injection of Aβ$_{1-42}$, had the same precipitating effect on tau pathology in a second mutant tau strain (Gotz *et al.,* 2001)

It was with the generation of a triple transgenic mouse (APP /tau/PSEN1) that a model that closely recapitulated human AD pathology became available (Oddo *et al.,* 2003). Furthermore Aβ deposition preceded tangle formation in this model supporting the amyloid cascade hypothesis.

The cascade hypothesis does not suggest a direct interaction between tau and Aβ but rather that an extracellular Aβ build up eventually results in altered neuronal tau kinase and phosphatase activity precipitating tau hyperphosphorylation (Hardy and Selkoe, 2002). However support for such a direct interaction came from *in vitro* studies where Aβ in the presence of tau forms fibrillar aggregates containing both molecules (Giaccone *et al.,* 1996). Of course such an interaction would require the intraneuronal build up of Aβ, a hypothesis that remains largely left field despite reasonable evidence supporting it (D'Andrea *et al.,* 2001; LaFerla *et al.,* 2007; Gouras *et al.,* 2010).

A particularly interesting finding was that 'neurotoxic' Aβ failed to cause degeneration of neuronal cultures from tau null mice (Rapoport *et al.,* 2002). In 2007 a similar effect was demonstrated in a mouse over-expressing human APP where behavioral deficits were attenuated on a tau null background (Roberson *et al.,* 2007). As tau did not reduce the levels of APP or Aβ the authors surmised that it must 'uncouple Aβ from downstream pathogenic mechanisms'. They were able to rule out Aβ-mediated modifications of tau such as

hyperphosphorylation or truncation as the mechanism. It seemed that tau was sensitising the mice to Aβ-mediated over excitation and potential excitotoxicity.

Excitotoxicity in the brain can result from overactivation of N-methyl-D-aspartate (NMDA) receptors (NRs) and it has been postulated that Aβ causes excitotoxicity through a NR-mediated mechanism (Snyder *et al.*, 2005). Under normal conditions the phosphorylation of these NRs by kinases such as Fyn facilitate interactions with post-synaptic density protein 95, the necessary conduit in the perpetuation of the excitatory signal. Interestingly increased Fyn expression was known to exacerbate toxicity in APP transgenic mice (Chin *et al.*, 2005). These factors all appeared unrelated until Ittner and colleagues showed in a dysfunctional tau mouse model, that when the tau-mediated dendritic transport of Fyn was prevented, Aβ-mediated excitotoxicity and premature lethality was attenuated (Ittner *et al.*, 2010).

It remains unclear how Aβ actually decreases the threshold for glutamate excitotoxicity but it is not thought to be through direct binding to the NRs (Snyder *et al.*, 2005). Nevertheless the NR antagonist, memantine is considered to act by blocking Aβ-neurotoxicity (Miguel-Hidalgo *et al.*, 2002).

4.4 Sporadic forms and the APOE ε4 genotype

There are very few factors, genetic or environmental, that have reproducibly been shown to modify the risk for sporadic forms of AD. Age, family history, female gender, low education, head injury and type II diabetes seem to increase risk. While long-term anti-inflammatory use and performing mental and physical activity seems to be protective. This area has been recently reviewed (Sutherland *et al.*, 2011b). The finding of AD prevalence being inversely associated with education led to the idea of non-demented aged individuals having a 'cognitive reserve' (Stern *et al.*, 1994). This hypothesis suggests that the more our brains are utilised the better they are able to withstand or perhaps more accurately compensate for, increasing AD pathology. Nevertheless, apart from ageing itself, all these factors have been shown to have very minor effects on AD risk.

One notable exception here is the possession of the *APOE* ε4 allele which could account for as much as 50% of the attributed risk in AD (Ashford, 2004). This effect was first described in 1993 (Corder *et al.*, 1993) but it is still not known how this common variant actually modifies disease risk. ApoE is the major apolipoprotein of the brain and its primary role is the delivery of lipids and particularly cholesterol to neurons from astrocytes. There are three major protein isoforms (ε2/3/4) based on their respective positions when separated by isoelectric focussing (IEF) (Zellner *et al.*, 2009). The variants are generated by two non-synonymous single nucleotide polymorphisms (SNPs) in exon 3 of the gene, rs429358 and rs7412. Alternative cytosine or thymine bases at these sites leads to either arginine or cysteine at positions 112 and 158 in apoE protein, respectively. The apoE ε4 isoform has arginine at both positions and varies from the common ε3 isoform in both its binding affinities (Weisgraber *et al.*, 1982) and degradation properties (Fukumoto *et al.*, 2003; Riddell *et al.*, 2008). The risk of sporadic AD increases with the dose of the APOE ε4 allele; heterozygotes are at a two-fold higher risk but ε4 homozygotes are at a greater than 12-fold risk (Corder *et al.*, 1993). There is a similar effect on the age at disease onset with an estimated decrease of 7-9 years per allele (Chapman *et al.*, 2001). However, possession of this allele is neither essential nor sufficient for AD. ApoE ε4 has an increased binding

efficiency for Aβ (Strittmatter *et al.*, 1993) and can accelerate Aβ fibril formation *in vitro* (Castano *et al.*, 1995). It is therefore generally interpreted that the apoE ε4 isoform modifies AD risk by accelerating the development of plaques although it is probably a more complex association between neuronal lipid content and amyloid precursor protein metabolism (Grosgen *et al.*, 2010).

Before the arrival of GWAs, a meta-analysis of single candidate gene studies showed that, apart from APOE, very few other gene loci modified AD risk and their effects were all very modest (odds ratios ~1.25) (Bertram *et al.*, 2008). The first GWAs in 2007 essentially confirmed these findings (Grupe *et al.*, 2007; Reiman *et al.*, 2007)

Subsequent GWAs would concentrate on defining the non-*APOE* genetic component of AD and in 2009 two independent studies reported an association with a second apolipoprotein-encoding gene, CLU, which encodes clusterin or apoJ and a gene called PiCALM (which encodes phosphatidylinositol binding clathrin assembly protein) (Harold *et al.*, 2009; Lambert *et al.*, 2009). Clusterin is best known as a chaperone protein but as its alternative name suggests it is also found in lipoprotein particles and regulates cholesterol and lipid metabolism in the brain (Nuutinen *et al.*, 2009). A feature of these later GWAs were their two-tiered approach where only SNPs deemed significant in a first cohort were tested in an additional independent cohort and only those replicated in both reported as significant. This strategy reduced the multidimensionality of the study and theoretically the risk of generating false positives. Lambert and colleagues also found an additional association with the CR1 gene, encoding a complement receptor (Lambert *et al.*, 2009).

In 2011 two further GWAs were reported that combined these multi-tier approaches with meta-analyses of additional data sets to further increase their detection sensitivity (Hollingworth *et al.*, 2011; Naj *et al.*, 2011). In combination these 2011 studies confirmed 10 significant loci associated with sporadic AD included the genes previously described (APOE, CLU, PICALM and CR1). One of the co-authors from these studies suggested that these 10 genes implicate three pathways in AD pathogenesis, immune system function, cholesterol metabolism and synaptic cell membrane processes (Morgan, 2011). He further suggested that these pathways appear to be largely Aβ-independent. However Aβ is known to inhibit synaptic activity and Aβ can bind to membranes where it may actually modulate the lipid make-up including cholesterol content (Grimm *et al.*, 2005).

Morgan considers that GWAs have now accounted for up to 50% of genetic risk in sporadic AD (Morgan, 2011). The question remains what has happened to the missing heritability? SNP arrays detect common variants (SNP directly and copy number variants by inference) but there is an alternative hypothesis that a large proportion of AD cases will result from rare variants in many different genes (Pritchard, 2001). Alternatively, Cooper and Shendure argue that it is not the GWAs technology that is limiting detection of common variants but rather our interpretation of the data (Cooper and Shendure, 2011). They maintain that an improvement in 'probability' is required before such studies will reach their maximum detection potential. This means that the analysis needs to be limited to functional entities only to reduce the number of tests carried out. They admit that defining which SNPs are functional (or in close linkage disequilibrium with such SNPs) remains a 'work in progress' but report steady advances in both computational approaches that predict changes in protein structure in non-synonymous SNPs and multiplex experimental approaches that combine mutagenesised libraries, *in vitro* transcription and RNA-Seq.

Two other factors potentially lowering the power of GWAs are not platform-related at all. As discussed above AD pathology is present in 30% or more of our age-matched controls and there are variations in the clinical presentation of AD. In particular it seems highly probable that individuals who develop clinical variations such as posterior cortical atrophy or logopenic aphasia will have different underlying genetic susceptibilities. It is hoped that biomarkers will be able to facilitate more focused comparisons of AD sub-groups or allow the dichotomous case-control paradigm to be replaced by an investigations of a continuous variable such as amyloid load using positive electron tomography (PET) (Sutherland *et al.*, 2011b).

4.5 Transcriptomics and brain tissue

Most readers will associate the term 'microarray' with whole genome expression studies. Microarray (expression) studies have been extensively used over the last decade for the analysis of human tissue and animal models. As discussed above, researchers were expecting a relatively high 40 to 100,000 protein-encoding genes in the human genome. This expectation was largely based on the evolutionarily complex human brain. However comparative genomic analyses have now shown that the increase in complexity and specialisation of the human brain is largely derived at the level of transcription (Enard *et al.*, 2002). This includes the utilisation of variable length 3' untranslated regions (Ramskold *et al.*, 2009) and alternatively spliced isoforms (Pan *et al.*, 2008; Wang *et al.*, 2008). This transcriptomic diversity will underlie both brain function and presumably dysfunction, making the transcriptomic analysis of postmortem human brain tissue a seemingly ideal experimental paradigm to find pathogenic clues in AD.

A meta-analysis of microarray studies in AD utilising brain tissue has not been undertaken but a review of reported finding suggests that these studies have been largely discordant and disappointingly have not provided novel clues about AD pathogenesis (Courtney *et al.*, 2010). These divergent findings may be due to a number of different factors. High RNA quality is the major factor in the subsequent quality of microarray data. Brain tissue pH is the major determinant of RNA quality but unfortunately it is the agonal period that is critical in determining pH, and this is both long in neurodegenerative disease and out of the control of the researcher. In comparison to animal or cell culture models RNA quality is invariably poorer from postmortem brain tissue (Preece and Cairns, 2003). The study of postmortem tissue is also inherently a retrospective analysis. The time component of neurodegenerative diseases means many rounds of tissue insult and compensatory host responses will have occurred, particularly in regions such as the entorhinal cortex in AD. The pathology in the postmortem brain may have little informative value on what precipitated the disease at the transcriptomic level. In other words it becomes impossible to delineate disease 'cause' from 'effect'.

Variability in microarray studies may also reflect limitations in the technology itself (Sutherland *et al.*, 2011a). A new NGS-based platform promises to address most of these issues and is discussed immediately below, but prior to this let us briefly look at a combination of the two main methodologies discussed above, GWA and expression studies. These can be combined to derive expression quantitative trait loci (eQTL). In a direct experiment approach genomic DNA of a case-control cohort will be assayed by SNP array while expression analysis will be carried out on say, RNA from peripheral leucocytes of the

same individuals. The expression of each individual transcript can be regarded as an independent variable and tested against all the SNPs on the GWAs array. This experiment can detect 'cis' effects of SNPs influencing the expression of their own gene but also 'trans' effects of SNPs that are spatially disparate from the gene of interest.

Alternatively an indirect approach can be used where the expression analysis is carried out in the brain tissue of neuropathologically confirmed cases and controls while the GWAs is in an independent AD cohort from (ideally) the same population. Such an indirect study has been carried out in AD and their major findings were three SNPs that associated significantly with IDE (insulin degrading enzyme) expression levels. This is a very interesting finding as IDE actually degrades Aβ (Kurochkin and Goto, 1994) and a meta-analysis, although not the latest GWAs, suggests that it is associated with sporadic AD (Bertram *et al.*, 2007). Presumably such studies will soon be reported with the partial or complete use of NGS.

4.6 RNA-Seq

As will be seen in the next section of this chapter NGS will influence nearly all aspects of research on disease pathogenesis. However it may have the greatest impact in trancriptomic analysis (Sutherland *et al.*, 2011a). In comparison to microarrays, RNA-Seq with its linear dynamic range and sensitivity for expression changes in low-abundant transcripts, provides a much more accurate (digital) signal. All transcripts can be confidently assumed to be present regardless of their level of expression. Second, microarrays rely on known genomic sequences for their probe design whereas RNA-Seq, with no such limitation, can detect novel transcripts (Cloonan and Grimmond, 2008). Third, microarrays generally quantify only the total transcripts for each gene but RNA-Seq, with its single base resolution, can detect the exact location of transcription boundaries allowing all transcriptional outputs to be quantified. This includes variants due to alternative promoter usage, splicing patterns and 3' UTR lengths that are unique to the human brain. A recent 'proof of concept' RNA-Seq study compared commercial RNA samples from AD patients with pooled control samples (Twine *et al.*, 2011). One of their major findings was the dysregulation of *APOE* transcription in the temporal lobe of an AD patient. SNPs in and around the APOE gene seemingly associate with AD independently of the ε4 effect, although there is a 7 kb linkage disequilibrium block that covers the entire APOE gene locus (Belbin *et al.*, 2007). It is anticipated that more RNA-Seq studies of postmortem brain tissue will appear in the literature in the near future.

4.7 Proteomics

The application of proteomics to AD has involved analyses of CSF, plasma and postmortem brain tissue. The driver for this research is to find biomarkers or a proteomic signature to improve AD diagnosis and potentially allow preclinical diagnosis (Zellner *et al., 2009*).

It has been known for sometime that there are protein-based alterations in the CSF profile of AD patients. Given the role of excess $A\beta_{1-42}$ in the disease it is perhaps surprisingly to see that this specific peptide is reduced in patient (clinical) analytes (Blennow and Hampel, 2003). This has been explained by the pathogenic retention of $A\beta_{1-42}$ in the parenchyma

facilitating plaque development. However there are increases seen in both phosphorylated tau species and total tau in AD patients' CSF.

A proteomic study that utilised two-dimensional gel electrophoresis (2-DE) found 23 differentially expressed proteins in the CSF of AD patients (Finehout *et al.*, 2007). These included a down-regulation of apoE (but they did not identify Aβ or tau). The sampling of CSF is relatively straightforward but it is not without risk of infection or spinal cord damage. There are also important ethical considerations in consenting dementia patients for such procedures although this issue also extends across the breadth of potential clinical research.

Researchers and clinicians are therefore keen to find brain-specific (and disease-specific) patterns of expression in serum or plasma samples that are more easily sampled and can be sampled repeatedly during the course of the disease. A disease-specific pattern in peripheral samples could reflect a number of scenarios; pathogenic species have drained or potentially leaked from the brain, there are systemic manifestations of the disease process or the functional consequences of underlying genetic susceptibility can be detected peripherally. As the brain is behind the relatively impermeable blood-brain barrier, leaked metabolites are unlikely to contribute greatly to plasma. Nevertheless the proteins, complement factor H and α-2 macroglobulin have been detected in plasma using by 2-DE and immunochemical assays although their sensitivity (62%) and specificity (60%) is far below those required for a biomarker (Hye *et al.*, 2006). In a recent study the reduced levels of apoE seen in the CSF, have also been seen in plasma of early stage AD sufferers, and particularly in *APOE ε4* carriers. The decrease in apoE levels was inversely correlated with Aβ load seen on PET (Gupta *et al.*, 2011).

5. Future

As we contemplate the future of research in AD, it is worthwhile reiterating that there are currently no treatments that slow the progression of the disease. At their first clinical presentation AD sufferers will already have significant neuronal loss. Ideally, neuroprotective agents are required but these would still be relatively ineffective if only implemented at the onset of clinical signs. A successful treatment regime is dependent on the co-discovery of a therapeutic target(s) and a preclinical biomarker(s). These, in turn, are both predicated on gaining a greater understanding of early pathogenic events.

5.1 Advances in understanding pathogenesis

Without doubt the major technological advance in biology is NGS. It seems only a matter of time before the much-anticipated '$1000 genome' is standard practice in both research and clinical practice (Pareek *et al.*, 2011). Furthermore NGS is about to become third generation sequencing where nucleic acids are sequenced without the need for preparatory PCR amplification of templates, a procedure that potentially introduces biases. These 'third-gen' technologies run at nanoscale proportions and rely on either detecting the exonuclease cleavage of a specific nucleotide from the template or their incorporation into a newly synthesised DNA strand. It is predicted that these new platforms will achieve read lengths of around 1000 base pairs making the subsequent alignment to a reference genome a more rapid and accurate process.

In terms of AD research, NGS will spell the end of the current design of GWAs. All study subjects will soon be sequenced directly allowing both the 'rare' and 'common variant' hypotheses to be simultaneously tested. We are already seeing an intermediate combination of SNP global analyses with more focused sequencing efforts (Lupton *et al.*, 2011). For example, an investigation of hypertriglyceridemic patients by GWAs followed by the resequencing of candidate genes uncovered a significant burden of rare variants (Johansen *et al.*, 2010).

It is interesting to postulate whether the knowledge of all genetic variation between affected and unaffected individuals (in a cohort) will deliver us the complete understanding of AD pathogenesis. There is no doubt that we will uncover hitherto unknown genetic clues that could increase our focus towards one defective pathway. However the single mutation in the autosomal-dominant inherited Huntington's disease was discovered in 1993 (The Huntington's Disease Collaborative Research Group, 1993) and much remains to be known about the functional ramifications of that defect.

In terms of transcriptomics, RNA-Seq templates usually involve polyA pre-selection so that only the mRNA fraction is sequenced however total RNA approaches are available where ribosomal RNA, which accounts for as much as 90% of the transcriptome, is selectively depleted. The move to total RNA approaches seems prudent given the increasing importance of non-coding and non-polyadenylated RNA species in biology (Mattick *et al.*, 2010) and the human brain in particular (Mattick, 2011). Non-coding RNA is transcribed from what was previously called 'junk' DNA and there is an amazingly diverse and continually expanding list of different RNA species being discovered. Perhaps more daunting is that single RNA molecules can also exist as structural variants with different functional roles (Wan *et al.*, 2011). Notwithstanding the latter, the RNA-Seq platform finally gives us the opportunity to comprehensively investigate the transcriptome of the human brain (Sutherland *et al.*, 2011a).

5.2 Diagnosis

When the first comprehensive criteria for clinical diagnosis were formulated in 1984, the only imaging modality in routine use in neurology was computer-assisted tomography. Neuroimaging has come a long way since then with volumetric magnetic resonance imaging (MRI), functional MRI including diffusion tensor imaging, single-photon emission computed tomography (SPECT) and PET with the amyloid-binding ligand, ^{11}C-Pittsburgh Compound B (PiB-PET) and brain glucose metabolism (FDG-PET)) all been applied to AD patients. Neuroimaging in AD has recently been the subject of a large international public/private partnership, based in the USA (Weiner *et al.,* 2010). Established in 2004, the Alzheimer's disease neuroimaging initiative (ADNI) seeks to validate MRI and PET images, in combination with CSF/blood biomarkers, as predictors and outcomes for use in clinical trials of AD treatments. They had a particular interest in establishing the criteria for preclinical AD and so recruited elderly controls and MCI patients, as well as AD sufferers, and followed them prospectively. ADNI consider, as per the amyloid cascade hypothesis, that the earliest detectable changes in at risk individuals are related to Aβ. Moreover there is a detectable initial increase in CSF $A\beta_{1-42}$ (preclinical) and amyloid deposition using PiB-PET. As discussed above these changes have been included in preclinical criteria but only

for use in a research context at this stage (Sperling *et al.,* 2011). When this detectable change in Aβ metabolism actually begins is unknown but decreases in CSF Aβ$_{1-42}$ are seen as early as the 6th decade in apoE ε4 carriers and their age-related decrease is more severe (Peskind *et al.,* 2006). Similarly PiB imaging of nondemented aged persons in Australia suggested that 33% of healthy controls had high PiB binding (Rowe *et al.,* 2010). Interesting a correlation between PiB binding and cognition was only seen in *APOE* ε4 carriers.

The increases in CSF phosphorylated and total tau are generally considered a later disease marker of the disease representative of neuronal degeneration, and likely to coincide with brain atrophy, hypometabolism or hypoperfusion on imaging (Weiner *et al.,* 2010). However at least one study of MCI suggests that by this stage there are already decreases in CSF Aβ$_{1-42}$ and increases in tau (Herukka *et al.,* 2005). Rowe and colleagues suggested that Aβ deposition (and increased CSF tau) are probably inevitable with ageing and other factors are involved in conversion to dementia (Rowe *et al.,* 2010).

5.3 Treatments in development

Although not the subject of this chapter it would seem remiss not to touch on the excitement currently generated by stem cell technology in medical research. The possibility of regenerative medicine in neurodegenerative disease has been stimulated by the demonstration of neurogenesis in the human adult brain (Eriksson *et al.,* 1998), the induction of stem cells from differentiated adult cells (Takahashi *et al.,* 2007) and the conversion of these cells to mature phenotypes such as dopaminergic (Soldner *et al.,* 2009) and motor neurons (Dimos *et al.,* 2008). Regenerative medicine or cell transplantation therapies may prove efficacious in many diseases although the chances in AD, with its extensive neuronal loss, do appear more remote. A therapy promoting endogenous neurogenesis looks more promising and its stimulation through physical activity or environmental enrichment seen in animal models may yet underlie the 'cognitive reserve' hypothesis. Furthermore the potential for modelling AD using patient-derived cell lines is an exciting field with applications in understanding basic pathogenesis through to preclinical drug screening and clinical toxicity studies (Sutherland and Sidhu, 2011).

The mechanism of action for the majority of drugs in current Phase II or III clinical trials is limiting the production or increasing the clearance of Aβ (Gravitz, 2011). These include antibodies to Aβ, inhibitors of Aβ aggregation and inhibitors of γ-secretase. In comparison inhibitors of β-secretase have proved more difficult to develop because of difficulties crossing the blood-brain-barrier and their poor affinity for the catalytic site, but two reports suggest that these roadblocks may have been solved (Atwal *et al.,* 2011; Yu *et al.,* 2011).

Two of the four drugs in Phase III trials, bapineuzumab and solanezumab are humanised monoclonal antibodies (derived from murine antibodies). These passive immunisation strategies were developed when initial studies testing an aggregated Aβ vaccine were abandoned due to 6% of the participants developing sterile meningoencephalitis (Orgogozo *et al.,* 2003). An Aβ vaccine had previously removed and prevented plaques in mutant APP transgenic mice, (Schenk *et al.,* 1999) and reversed their learning deficits (Janus *et al.,* 2000; Dodart *et al.,* 2002). Passive immunisation seemed to have a similar effect in mice but the

earliest reports from human trials suggest that, despite reducing amyloid load, these strategies have no effect on cognitive function (Rinne *et al.*, 2010). Furthermore trials determining the efficacy and safety of the γ-secretase inhibitor, semagacestat, had to be terminated due to actual cognitive decline (Cummings, 2010).

These results have been seen by some as serious threats to the validity of the amyloid cascade hypothesis in sporadic AD, although proponents have countered these criticisms by arguing that Aβ therapy is failing because it is instituted too late in the disease process (Gandy, 2011; Golde *et al.*, 2011). They maintain that Aβ therapy needs to be instituted as a prophylaxis and this, of course, means the preclinical identification of future AD patients and the treatment, or at least testing, of individuals without symptoms (Golde *et al.*, 2011).

According to Golde, Schneider and Koo, and based on the findings from imaging and CSF biomarker studies described above, individuals destined to have AD show detectable Aβ deposition at least 10 years earlier. Furthermore amyloid plaques visualized by radioligand imaging and low CSF Aβ$_{1-42}$ can be monitored throughout potential trials of Aβ therapies. It is not clear how an original cohort might be selected but aged *APOE* ε4 carriers were suggested for screening (Golde *et al.*, 2011). This hypothesized approach raises some very interesting ethical questions, not to mention changes required to regulatory criteria that are currently based on clinical symptoms.

5.4 Preventive health

Epidemiological studies were instrumental in establishing the connection between serum lipid levels, particularly high cholesterol, and coronary heart disease. Proponents of prophylactic Aβ immunotherapy might argue that their proposal is similar to the current use of statins in reducing blood cholesterol. However epidemiological studies have been relatively unsuccessful in finding factors that modify the risk for AD, meaning that any proposed preventative strategies remain speculative at best.

The statins story is an interesting one because individuals in the initial trials for heart disease appeared to have a reduced incidence of AD (Jick *et al.*, 2000; Wolozin *et al.*, 2000). This led to considerable research into cardiovascular risk factors in AD including hypertension, elevated serum cholesterol, smoking middle age obesity and type II diabetes. Interesting only type II diabetes survives meta-analyses (Sutherland *et al.*, 2011b).

Remaining physically and mentally active does seem to be relatively effective but it is likely that a cognitive reserve allows individuals to starve off the effects of AD pathology rather than decreasing the pathology itself. Of course this would still be an effective method to reduce the societal burden of AD.

Despite all the research, the current situation in AD could still be summarised by the following paragraph from a recent commentary in the Journal supplement, Nature Outlook:

"A big part of the problem is that researchers don't know enough about the biology of Alzheimer's disease to identify the right targets. The disease is the result of a long chain of events, but some of the links in that chain are still a mystery − nobody is certain which link to cut to stop disease progression" (Gravitz, 2011).

6. Conclusion

Given the passage above the reader may become overly pessimistic about the possibilities for a cure of Alzheimer's in the immediate future. In its general use the term 'cure' implies that there is a readily identifiable agent that causes AD and that this agent can be attenuated or even removed without any serious deleterious effects to the sufferer.

All AD research to date suggests that there is no clear single aetiology for the majority of sufferers. The major pathogenic hypothesis is that in susceptible individuals, on a background of ageing, there is an increased production or decreased clearance (Mawuenyega *et al.*, 2010) of a normal 'degradative' product called Aβ whose build up in the brain becomes neurotoxic.

An alternative or adjunctive interpretation is that Aβ has a physiological role in the brain, perhaps synaptic pruning or inhibiting excitatory activity and that Aβ metabolism itself becomes dysregulated in response to an unknown aetiological factor. The individuals who develop AD may have a greater propensity to deposit Aβ or are less resistance to its downstream effects. In the latter scenario, monogenic forms of AD could still explained by a greater propensity to oligomerisation and deposition. The caveat is that prophylactic Aβ immunotherapy may prove to have more serious side effects than if Aβ is just a degradative product.

The amyloid cascade hypothesis is a very good one that no doubt encapsulates important aspects of the pathogenesis of sporadic AD. However technological advances such as GWAs suggests that Aβ-independent mechanisms are (also) important in AD while RNA-Seq is only now offering a methodology where the transcriptome of the human brain can actually be comprehensively examined. It therefore seems too premature to place all our therapeutic eggs in the Aβ cascade basket.

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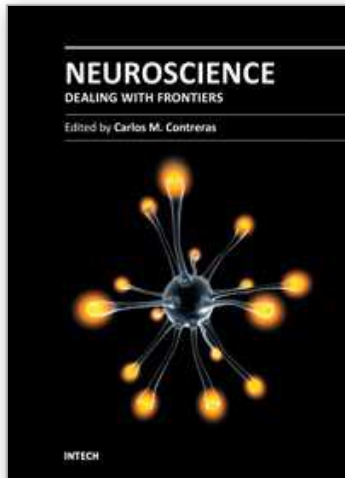
Neuroscience - Dealing With Frontiers

Edited by Dr. Carlos M. Contreras

The Neuronal Doctrine recently reached its 100th year and together with the development of psychopharmacology by the middle of 20th century promoted spectacular developments in the knowledge of the biological bases of behavior. The overwhelming amount of data accumulated, forced the division of neuroscience into several subdisciplines, but this division needs to dissolve in the 21st century and focus on specific processes that involve diverse methodological and theoretical approaches. The chapters contained in this book illustrate that neuroscience converges in the search for sound answers to several questions, including the pathways followed by cells, how individuals communicate with each other, inflammation, learning and memory, the development of drug dependence, and approaches to explaining the processes that underlie two highly incapacitating chronic degenerative illnesses.

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