Chapter 1

The Contribution of Microscopy to the Study of Alzheimer's Disease, Amyloid Plaques and Aβ Fibrillogenesis

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Abstract: A broad survey is presented in this chapter, dealing with the impact that microscopy has made to the study of Alzheimer's disease, amyloid plaques and amyloid-β fibrillogenesis. This includes classical light microscopy and the modern immunolabelling and confocal microscopies, together with the contribution of transmission electron microscopy and atomic force microscopy. Whilst usefully standing alone, the individual microscopies often contribute most effectively when they are integrated with cellular, biophysical and molecular approaches.

Key words: Amyloid-β, Aβ, diffuse plaque, senile plaque, light microscopy, electron microscopy, atomic force microscopy, oligomer, fibril, fibrillogenesis.

1. INTRODUCTION

Since the initial post-mortem definition of cerebral amyloid plaques in the brains of ageing demented patients by Alois Alzheimer in the early 1900s from light microscopical (LM) observations, tremendous advances have occurred. Today much greater microscopical precision is now applied to brain intracellular neurofibrillary tangles and the extracellular diffuse and dense-cored/senile amyloid plaques; emphasis will be placed primarily on extracellular amyloid throughout this chapter.

Whole brain non-invasive 3D neuroimaging by Roentgen/X-ray computed tomography, magnetic resonance imaging and single

photon/positron emission computed tomography has recently provided useful information enabling the early diagnosis and progression of Alzheimer's Disease (AD) to be assessed at the gross anatomical and histological level (Heckl, *et al.*, 2004; Klunk *et al.,* 2003; Masdeu, 2004; Mathis *et al.,* 2004; Petrella *et al.*, 2003). Wadghiri *et al.* (2003), using transgenic (Tg) mouse animal models for AD presented a method to detect Aβ plaques by whole brain magnetic resonance microimaging (microMRI), correlated with immunohistochemistry. This technique may well be of future significance for the detection of plaques in AD patients. Similarly, the use of radioiodinated thioflavine derivatives as tracers for imaging amyloid plaques by autoradiography holds considerable potential (Kung *et al.*, 2003). Such approaches cannot be considered further here. Rather, it is my intention to emphasise the contribution of light microscopical (LM) imaging, using conventional tissue staining and specific staining for amyloid deposits, and then lead on to contribution made by transmission electron microscopy (TEM) and atomic force microscopy (AFM) at the cellular and macromolecular level.

A distinction can be made between AD studied *in vivo,* with samples taken from man or an animal and then studied experimentally *ex vivo*, such as during cell culture or biochemical analysis, with experiments that are more truly *in vitro* because they use purified cloned proteins or chemically synthesised peptides. Whilst for most researchers the simple distinction between *in vivo* and *in vitro* is often sufficient, the recent use of the term *ex vivo* sometimes provides a useful element of precision for the description of some experimental systems.

Although most LM studies were initially performed on human autopsy samples, increasingly LM is being applied to the brains of animal AD models (*e.g.* gene knock-out and transgenic mice) and neuronal tissue in cell culture. The newer techniques of LM, such as fluorescence, confocal and multiphoton imaging are also increasingly being used and provide a meaningful continuity from the earlier LM data, now spanning almost a 100 years of AD research.

TEM has also made a useful contribution to studies on post-mortem brain samples from AD patients and Tg animal AD models, by providing supportive evidence for the existence and structure of extracellular diffuse and compact neuritic/senile plaques. With the increasing availability of synthetic amyloid-β (Aβ) peptides (*e.g.* 1-40, 1-42 and 1-43) and smaller fragments thereof, together with Aβ mutants and their synthetic equivalents, several studies relate to Aβ fibrillogenesis and the subsequent investigation of fibril stability, drug and protein interaction. In parallel with TEM studies on Aβ, a wealth of generally supportive structurally relevant information has come from studies on other amyloid systems, where pathological

protein/peptide alteration involves a transformation of an initial α-helix-rich native soluble peptide into an unfolded crossed β-sheet-rich insoluble fibril-forming peptide. Usually, this related literature will only be commented upon briefly. The full impact of TEM immunolabeling for the study of amyloid plaque, *ex vivo* fibrils isolated from brain and *in vitro*-formed fibrils from synthetic Aβ peptides has yet to be seen; more extensive single and double immunolabeling studies can be expected in the future.

With the ready availability of commercially constructed equipment and the increasing expertise in its use, atomic force microscopy (AFM) and the related scanning tunnelling microscopy (STM) have been increasingly applied to the study of Aβ oligomers, fibrils and fibrillogenesis over the past ~10 years. The data produced does in general conform well with and extend from that produced by TEM, but the possibility of directly performing dynamic experiments on oligomer formation and fibril polymerisation, and drug-induced disruption of these events, makes this microscopical technique extremely valuable.

The contribution of TEM and AFM in this field cannot be considered in isolation from the range of available biophysical, biochemical and biological techniques. When appropriate, some discussion of these additional approaches will be included. Often researchers utilize and integrate a number of approaches, with TEM sometimes representing only a minor experimental component that fails to do full justice to the possible contribution of this technique. Because of the diversity of the content of this chapter, it can to some extent be considered as an introductory survey to much that will appear in the subsequent chapters.

2. LIGHT MICROSCOPY

2.1 Background

Light microscopy has been widely used for investigations into Alzheimer's disease since the earliest studies in this field. In this chapter, only aspects relating directly to the formation and properties of Aβ-containing diffuse and senile plaques will be emphasized, leaving many other significant LM applications unmentioned. LM studies have utilized human (postmortem) and animal brain samples as well as cultured cells (neurones, microglia). Recently, studies using Tg animal models for AD (mice and *Caenorhabditis elegans*) have made a strong impact, in many cases using LM alongside cellular and biochemical approaches.

Conventional LM with hematoxalin and eosin (H&E), silver and Congo red staining, as well as fluorescence microscopy with Congo red,

thioflavine-S/T, an improved lipophilic thioflavine-T derivative (Kung et al., 2002) and dimethylamino-fluorenes (Lee *et al.*, 2003), fluorescein- or biotin-conjugated antibodies to Aβ and other components of the extracellular amyloid plaques and immunoperoxidase labeling, have all been successfully used. Advanced double-labeling immunofluorescence microscopy and confocal microscopy are making an increasing contribution. Classification of the progressive plaque formation in brain has been achieved by LM. Initially, immature/primitive plaques represent a loose accumulation of Aβ surrounded by abnormal neurones. Diffuse plaques are composed of an amorphous (non-fibrillar) or finely fibrillar accumulation of Aβ. Such diffuse plaques tend to stain only lightly with Congo red and thioflavine-S, but stain strongly with antibodies to Aβ. Mature/neuritic/senile plaques, which stain strongly with Congo red and thioflavine-S, tend to have a dense core of fibrillar amyloid surrounded by reactive astrocytes (macroglia) but no abnormal dystrophic neurones. Microglial (macrophage) cells are usually associated with all plaque types. Alzheimer senile plaques are found to be distributed throughout the cerebral cortex, cerebellum, amygdala, substantia innominata, hypothalamus, claustrum, tegmentum of the midbrain and rostral pons. In addition, senile plaques often occur adjacent to cerebral capillaries or larger vessels that have accumulated Aβ within their walls (cerebral amyloid angiopathy). These extracellular structural features can be detected by LM in the brains of non-demented persons, with or without head injury, but in a more pronounced manner in individuals suffering from AD, Down's syndrome (DS) and Creutzfeldt-Jacob disease (CJD) (Mochizuki *et al.*, 1996). A useful grading system for AD lesions has been advanced by Metsaars *et al.* (2003), within which the chronological sequence of neocortical lesions was graded 1 to 4, based on H&E and Congo red histology, and immunolabeling with anti-tau, -Aβ8-17 peptide, -APP, -ubiquitin and -cathepsin D. Immunohistochemistry was performed using biotinylated secondary antibodies and the streptavidin-biotin-peroxidase method, and the LM data was assessed by morphometric methods to minimize any subjective interpretation.

2.2 Application of light microscopy to cellular and extracellular studies on Aβ

Despite increasing claims that oligomeric forms of Aβ are responsible for nerotoxicity (*e.g.* Lambert *et al.*, 1998), an extensive literature exists, often including LM studies, showing that fibril formation may be important (Lorenzo and Yankner, 1994). Thus, in the present state of knowledge, it is best to keep a relatively open mind; both aspects will be duly mentioned within this chapter.

The prime location of Aβ oligomerization and fibril polymerization, *i.e.* cellular (neuronal/non-neuronal) or extracellular, has not been finally decided. Blood platelets are accepted as a major source of soluble plasmatic Aβ peptide, but it is not certain that the platelet-derived peptide is a principal source of brain Aβ plaques. Al-Khan (2002) discussed the concept that microglial cells may be responsible for the cellular processing of Aβ, via their lysosomal compartment. This was supported by EM studies of Weigel *et al.* (2000; 2001b) (*see below*). Accumulation of the amyloid precursor proten (APP) inside multivesicular bodies in cultured human brain pericytes, leptomeningal smooth muscle cells and vascular endothelial cells was shown

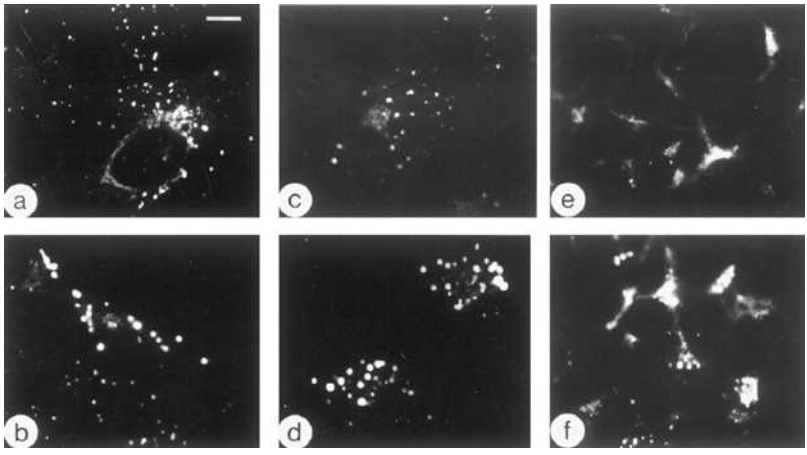

Figure 1. Immunofluorescence staining of untreated (A,C,E) and IFN-γ treated (B,D,F) cultures of human brain pericytes (A,B) human leptomeningeal arterial smooth muscle cells (C,D) and human microvascular endothelial cells (E,F) with anti-APP MAb P2-1. Large APP-positive intracellular organelles are present in all cell types after exposure to IFN-γ. Bar = 3 μm. Reproduced, with permission, from Verbeek *et al.*, Accumulation of the amyloid-β precursor protein in multivesicular body-like organelles. *Journal of Histochemistry & Cytochemistry* 50: 681-690, 2002.

using LM (Figure 1), including elegant double labeling immunofluorescence and EM by Verbeek *et al.* (2002). They suggested that multivesicle bodies function as an intermediate location in the intracellular trafficking of APP. That microglia could be involved in the clearance of exogenous Aβ1-42 was advanced by Takata *et al.* (2003), together with the fact that this cellular activity was facilitated by heat shock protein-90 (Hsp90). Furthermore, accumulation of Aβ1-42 within astocytes and the subsequent formation of astrocytic amyloid plaques in AD brains was shown using impressive LM immunohistochemistry by Nagele *et al.* (2003). Exclusive localization of Aβ in small astrocytic plaques to the subpial portion of the molecular layer of

the cerebrocortex and co-localization with glial fibrillary acidic protein are features which distiguish these plaques from those derived from neurones.

Activation of microglial cells within amyloid plaques appears to be associated with an inflammatory response. Lue *et al.* (2001) cultured microglia from post-mortem brain tissue from AD and non-demented (ND) individuals. They showed significant differences between AD and ND individuals with respect to the secretion of M-CSF and complement C1q. Although microglia are phagocytic there is no evidence for the internalization of extracellular oligomeric or fibrillar Aβ or for ingestion and loading with cell fragments and cholesterol-rich membranes from dead neurones, as occurs in inflammatory macrophage/foam cells associated with circulatory atherosclerotic plaques.

Others implicate neuronal cells in the formation of amyloid plaques. Wang *et al.* (2002) suggested that the α7 nicotinic acetylcholine receptor could be involved in Aβ accumulation in Purkinje cells and the formation of cerebrellar diffuse amyloid plaques. Whether the associated neuronal lysis could also account for the presence of significant quantities of membrane-derived cholesterol in the amyloid plaques was not addressed. However, it is abundantly clear that such cholesterol is derived from cholesterol synthesized within brain tissue rather than from the blood plasma. An interesting although not widely appreciated fact is the link between AD and cataract formation in the lens. Goldstein *et al.* (2003) have found that in AD individuals Aβ accumulates as electron dense deposits in the cytoplasm of supranuclear cortical lens fibre cells of AD individuals. These deposits contain Aβ immunoreactivity and show Congo red birefringence. *In vitro* fibril assembly studies showed that Aβ bound to αB-crystallin and promoted aggregation of this lens protein.

Application of LM to the study of Aβ in cerebellar plaques was performed by Tamaoka *et al.* (1995). The detection of Aβ1-42 and Aβ1-43 as major components of diffuse-initial phase plaques implied the importance of these two Aβ species in AD pathogenesis. Immunohistochemical analysis of hippocampal sections from AD brains by Shimizu et al. (2002) combined with dot and Western blotting and *in vitro* Aβ peptide aggregation indicated that acceleration of spontaneous isomerization of the Asp23 might be significant for the formation of fibrillar amyloid in sporadic AD and that mutations or modifications of Glu22 and Asp23 could play a pathogenic role (Figure 2). The advanced LM technique of laser confocal microscopy was used by Dickson and Vickers (2001) to define morphologically the characteristic substructure of diffuse, fibrillar and dense-cored Alzheimer plaques. In this work, the plaque-associated dystrophic neurites labeled strongly for tau and neurofilament proteins.

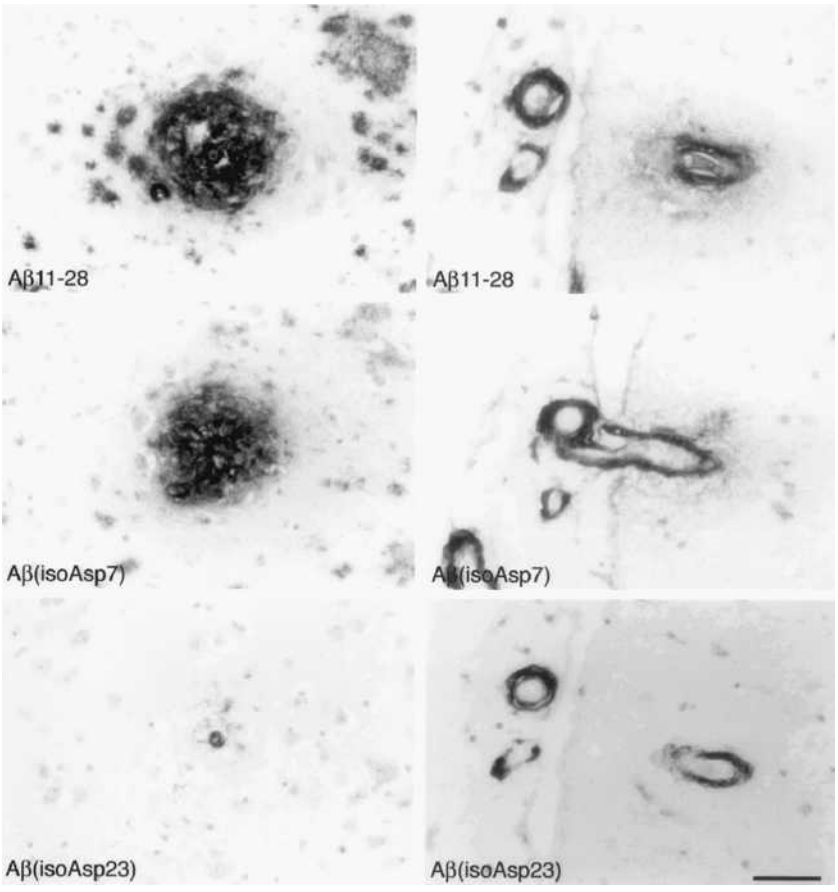

Figure 2. Immunohistochemical analysis of AD brain. Positively stained senile plaques and amyloid-bearing vessels in hippocampal sections. Bar = 50 μm. Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. from Shimizu *et al.*, Isoaspartate formation at position 23 of amyloid beta peptide enhanced fibril formation and deposited onto senile plaques and vascular amyloids in Alzheimer's disease. *J.Neurosci. Res.* 70: 451-461 © 2002.

From a detailed morphometric LM analysis of elderly non-demented and AD subjects Wegiel *et al.* (2001a) concluded that even at the end stage of AD the process of fibrillar plaque formation persists. Using quantitative immunohistochemical analysis activation of the Aβ-degrading endopeptidase, neprilysin (a zinc metalloproteinase), has been found to be reduced during aging of mouse hippocampus (Iwata *et al.*, 2002). This Down's regulation of neprilysin was considered to be relevant to AD

pathology and to Aβ deposition during normal aging. Using a similar approach with transgenic AD-like mice, Apelt *et al.* (2003) maintained that neuronal neprilysin Down's-regulation was accompanied by up-regulation in astroglia associated with Aβ-containing plaques. The up-regulation of neprilysin following gene transfer can be considered to be one possible route for gene therapy in AD (Marr *et al.*, 2003).

The alternative theory, that small Aβ oligomers are responsible for neuronal death centers on the direct action of oligomers on neuronal membranes and on the channel hypothesis. This latter concept has received support from the Aβ-induced unregulated calcium entry into neurones via calcium channels (Kawahara and Kuroda, 2000), resulting in cell death. Intracellular calcium fluorescence was quantified and correlated with loss of cell viability (*see also* Chapter 17).

2.3 Down's syndrome

Down's syndrome (DS) individuals have an extra copy of chromosome 21, which carries the gene for APP. Among the several clinical abnormalites of DS are mental retardation, dementia and development of AD-like symptoms, often at a relatively young age. Indeed, in this respect DS subjects can almost be considered to be natural transgenic individuals, as they express a greater quantity of brain APP and Aβ. The age-dependent progression of AD-like features in DS subjects was demonstrated immunohistochemically by Hirayana *et al.* (2003). They showed Aβ1-42 and Aβ1-43 accumulation in cortical neurones, but with Aβ1-40 in the cores of senile plaques. Neuronal damage was believed to be due to Aβ1-43 accumulation, and this was advanced as an explanation for the formation of neurofibrillary tangles. In an impressive application of LM and EM, Wegiel *et al.* (2002) correlated the development of vascular fibrosis and calcification in the hippocampus of normal, DS and AD individuals. Although AD subjects showed marked hippocampal vascular fibrosis, this was significantly less in DS subjects. An age-dependent association of the first complement component C1q with thioflavine-S positive mature plaques in hippocampus and frontal entorhinal cortex of DS subjects was shown using immunohistochemistry by Head *et al.* (2001). Neurones were also C1q positive and were associated with activated microglia. This again implicates inflammation in the development of AD neuropathology.

2.4 Transgenic animals

Although early transgenic (Tg) mouse models for β-amyloidosis were not fully equivalent to the pathology of AD, Walker *et al.* (2002) considered that

seeding of Aβ *in vivo* by injection of AD brain extracts into mouse brain might potentiate the pathological similarities. Indeed, Kuo *et al.* (2001) maintained that SDS-solubility of amyloid plaques from Tg mice was much greater than AD plaques. This was apparently accounted for by a reduced level of post-translational modification, such as N-terminal degradation, isomerization, racemization, pyroglutamyl formation, oxidation and covalent

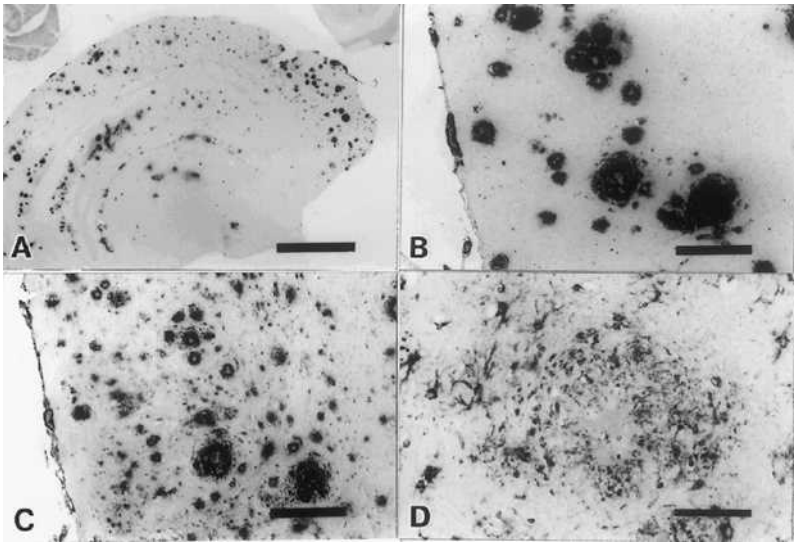

Figure 3. Amyloid deposits in the Tg2576 mouse brain. Senile plaques with large dense cores and amyloid angiopathy in 23-month-old transgenic mice visualized by Aβ1 (a), Aβ40 (b) and Aβ42 (c) antiboides. Note astrogliosis in close proximity to the dense-cored plaques in 20-month-old Tg2576 mice visualized using double staining Congo red and glial fibrillary acidic protein immunohistochemistry (d). Bars = 1 mm (a), 200 μm (b,c) and 50 μm (d). Reprinted, with permission, from Sasaki *et al.*, Amyloid cored plaques in Tg2576 transgenic mice are characterized by giant plaques, slightly activated microglia, and the lack of paired helical filament-typed, dystrophic neurites. *Virchows Arch.* 441: 358-367, © Springer-Verlag 2002.

linkage of Aβ dimers, required to fully stabilize the fibrillar Aβ within transgenic mouse plaques. Nevertheless, when brains from transgenic mice carrying the human APP (Swedish mutation) were compared with AD brain samples by LM immunohistochemistry and EM (Sasaki *et al.*, 2002), extremely large cored plaques were found in the older mice (18-29 months), which also contained slightly activated microglia (Figure 3). With a triple-transgenic AD mouse model (for APP, presenilin 1 and tau) Oddo *et al.*

(2003a) attempted to clarify the pathogenic role of intraneuronal Aβ in synaptic plasticity, within the context of both Aβ plaques and neurofibrillary tangles. In a further study, using the same triple transgenic model, (Oddo *et al.*, 2003b) concluded that amyloid deposition precedes tau tangle formation. An impressive combination of behavioural, and light and electron microscopical studies by Richardson *et al.* (2003), using the TAS10 Tg mouse line, have shown that definable changes precede amyloid deposition. Elegant LM immunohistochemistry of the hippocampus and entorhinal cortex using the PDAPP Tg mouse model, with 3D reconstruction of Aβ deposition, enabled Reilly *et al.* (2003) to differentiate the deposition of diffuse Aβ and compact plaques. A firm link between inflammation, assessed by the presence of activated astrocytes around plaques, and the early deposition of Aβ in the brain of TgCRND8 mice, was advanced by Dudal *et al.* (2004). The low molecular mass heparin, enoxaparin, has been found to reduce the size of Aβ deposits in the brains of Tg mice (Bergamaschini *et al.*, 2004) and at the same time markedly reduced the number of surrounding activated astrocytes.

Methoxy-X04 is a lipophilic derivative of Congo red, and has been used to label Aβ fibrils in AD plaques and in Tg mice. Systemic administration of methoxy-X04 enabled production of fluorescent images from the brains of living transgenic mice by multiphoton microscopy, thereby revealing individual plaques (Klunk et al., 2002). Furthermore, these authors proposed that methoxy-X04 might be suitable as an *in vivo* amyloid imaging agent in AD patients using photon emission tomography.

A doubly Tg mouse model, prone to both atherosclesosis and AD has been established by Li *et al.* (2003). Significantly, these mice show aortic atherosclesosis and cerebral β-amyloidosis, both conditions being enhanced by a lipid/cholesterol-rich atherogenic diet, along with impairment of learning capacity. Such considerations correlate with the views of de la Torre (2004), who believes that AD is more closely related to vascular disease than is commonly thought.

Transgenic *Caenorhabditis elegan* has been engineered to express human Aβ, produce amyloid deposits that stain with Congo red and thoflavin-S (Fay et al. (1998). In this animal model system for AD, $Leu^{17}Pro$ and $Meth^{35}Lys$ substitutions blocked the formation of thioflavine-S-reactive deposits, indicating an inability of Aβ to polymerize. The fluorescent, amyloid-specific dye X-34 (also a Congo red derivative) enabled *in vivo* staining of amyloid deposits to be monitored over a period of time (Link *et al.*, 2001). Transgenic *C. elegans* expressing a non-fibrillar Aβ peptide variant did not stain with X-34, but was positive with anti-Aβ antibodies. *Drosophila* expressing human Aβ1-40/42 have also been uses as a potential model for AD. Iijima *et al.* (2004), by immunostaining, showed the distribution of Aβ

deposits in the fly brain, with late onset of neuronal loss the the Aβ1-42 brains.

Some further consideration of the increasing use of transgenic animals will be given elsewhere in this chapter (section 2.5 and 2.6, and see also Chapter 2). However, the fact that all mouse AD Tg models are by no means equal, as emphasized by Lehman *et al.* (2003), must not be lightly overlooked. Furthermore, Schwab et al. (2004) considered that Tg mice overexpressing Aβ are an incomplete model for AD.

2.5 Oxidative stress

The possible involvement of reactive oxygen species (ROS) in the deposition of fibrillar Aβ and the neurotoxicity of Aβ is a concept that has been discussed for several years (Behl, 1999). As with other Aβ-related topics, LM continues to make an important contribution in this area. McLellan *et al.* (2003) have used both the AD Tg mice model and *ex vivo* human AD brain tissue for investigations on ROS. By multiphoton microscopy for the detection of oxidised fluorogenic compounds they showed that only dense cored/senile plaques produced oxygen free radicals. This supported the earlier claims of Matsuoka *et al.* (2001) using more conventional LM for the detection of the natural oxidative stress markers 3-nitrotyrosine and 4-hydroxy-2-nonenal. However, it was maintained by Drake *et al.* (2003), using the Tg *C. elegans* Alzheimer model, that oxidative stress precedes fibril formation. Because of the ability of Aβ to bind metal ions (Cu/Zn/Fe/Al) it is likely that oxidative events in SP cores should not be considered in isolation from the chemical properties of these metals and the way they may mediate oxidative damage promoted by Aβ (Dong *et al.*, 2003). Although Aβ neurotoxicity, apparently not mediated by hydrogen-peroxide, was implied by the study of Saito *et al.* (2001), a direct potentation of Aβ fibrillogenesis *in vitro* by hydrogen peroxide has recently been observed (*see below*, **3.3.3**). Immunocytochemical evidence for impairment of several endogenous enzymic antioxidant systems in brain by Aβ infusion has been demonstrated by Kim *et al.* (2003). That oestrogen and related compounds might be beneficial antioxidants is under intensive investigation (Behl and Mossmann, 2002), (*see also* Chapter 3).

2.6 Senile plaque-associated proteins

Several proteins have been found to associate with fibrillar Aβ within senile/dense cored plaques; they are often not present to the same extent within diffuse plaques. As already mentioned (see **2.2**, above) lens αB-crystallin is one of these proteins; others are acetylcholinesterase (AchE) the

apolipoprotein epsilon 4 genotype ($ApoE_4$), Apolipoprotein J (ApoJ; clusterin), α1-antichymotrypsin, heparan and chondriotin sulphate proteoglycans, catalase, complement C1q, the two pentraxins serum amyoid P component (SAP) and C-reactive protein (CRP), the complement receptor CD11b, glial fibrillar acidic protein (GFAP), cystatin C, vascular endothelial growth factor (VEGF) and others. In most cases LM has contributed significantly to the assessment of presence and localization of these Aβ-associated proteins in senile plaques, but this was not the case for catalase, where Milton (1999) found the affinity of this anti-oxidant enzyme for Aβ on microplates.

The $ApoE_4$ genotype has been shown by LM to be linked to the formation of classic/senile AD plaques in preference to diffuse plaques (Oyewole *et al.*, 1999). Also, immunohistochemicaal localization of apolipoprotein E in cerebral blood vessels in association with amyloid deposits was demonstrated by Navarro *et al.* (2003). Thioflavine-S-reactive plaques in doubly transgenic mice expressing APP and human AchE (Rees *et al.*, 2003) were found to be formed more rapidly than in singly APP transgenic mice. AchE was shown histologically to be associated with the plaques and thus it was suggested that it might play a role in the pathogenesis of AD. Indeed, the C-terminal region of acetylcholinesterase has been implicated in amyloid fibril formation (Cottingham *et al.*, 2003). That cholinergic dysfunction is a feature of AD was emphasized by the study of Luth *et al.* (2003). These workers used LM and TEM of thin sectioned brain to demonstrate choline acetyl transferase immunoreactivity of nerve fibres in the vicinity of Aβ-containing plaques.

Boyett *et al.* (2003) found that when complement C1q was injected into the brains of doubly transgenic APP, PS1 mice, it resulted in an increase number of Congo red staining fibrillar deposits. This *in vivo* potentiation of amyloid fibrillogenesis agreed with earlier *in vitro* data showing that C1q promoted Aβ nucleation and aggregation (Webster *et al.*, 1995). Aβ1-42 induced activation of microglia by the plaque-associated proteins C1q and SAP was shown by Veerhuis *et al.* (2003); the pro-inflammatory cytokines IL-1α, IL-1β, IL-6, and TNFα were all increased. These authors suggested that microglial activation precedes the neurodegenerative changes associated with AD. A generalized role for the pentraxins (SAP and CRP) in inflammatory diseases, including AD, was advanced by McGeer *et al.* (2001), leading to the concept that the pentraxins could be targets for therapeutic intervention, as expanded upon by Pepys *et al.* (2002) within the context of peripheral amyloidosis rather than for amyloid-β plaques in the brain.

2.7 Cholesterol in amyloid plaques

Accumulation of cholesterol in dense-cored fibrillar plaques in AD and transgenic APP (SW) mice was shown initially by Mori *et al.* (2001), using fluorimetric staining for filipin and enyzmically using cholesterol oxidase. This work was extended by Burns *et al.* (2003), who showed co-localization of cholesterol and ApoE in hippocampus of transgenic PS1/APP mice (Figure 4). Data from both these groups showed that cholesterol was not associated with diffuse plaques, indicating Aβ fibril-specific cholesterol binding (*see also* section **3.3.5**).

It is known that the epsilon 4 isoform of ApoE is a predominant genetic risk factor in both familial and sporadic AD (reviewed by Poirier, 2003). The binding and transport of cholesterol by $ApoE_4$ to microglia and astrocytes may provide a cellular pathway for non-esterified cholesterol to be recovered from injested neuronal membranes, released and transported to amyloid plaques. It is likely that cholesterol is bound to fibrils in a molecular or micellar form rather than a microcrystalline form (as is known to occur in atherosclerotic plaques, in association with calcium phosphate). Also, conversion of brain cholesterol to 24S-hydroxycholesterol, due to the presence of a microglial oxidative cytochrome P-450 (termed CYP46), was advanced by Bogdanovic *et al.* (2001) as a significant factor in relation to the mechanism for cholesterol efflux from microglia.

Using LM immunohistochemistry and image analysis Pappola *et al.* (2003) assessed the amyloid load in human brain and correlated this with the presence of cholesterolemia. They were able to conclude that serum hypercholesterolemic could be an early risk factor in AD development, but from older subjects their data was not clear-cut (the data may have been biased by deaths due to cardiovascular disease). Conclusive evidence for the link between CSF cholesterol and brain amyloid has come from the genetic study of Wollmer *et al.* (2003) who linked the incidence of polymorphism of the gene encoding acyl-coenzyme A: cholesterol acyltransferase with a low CSF cholesterol level and low brain amyloid load.

However, a note of caution comes from the work of Park *et al.* (2003) who showed that in female transgenic mice, lovastatin actually enhanced Aβ production and senile plaque deposition. Nevertheless, the cholesterol-fed rabbit has been used as a model system for AD (*e.g.* Sparks, 1996). Furthermore, that trace quantities of copper are required for the formation of amyloid plaques in cholesterol-fed rabbits has been shown by Sparks and Schreurs (2003), using immmunohistochemistry to define senile plaques. The link between cholesterol and iron-dependent oxidative damage in both atherosclerosis and neurodegeneration has been reviewed by Ong and Halliwell (2004), with the conclusion that this might explain why

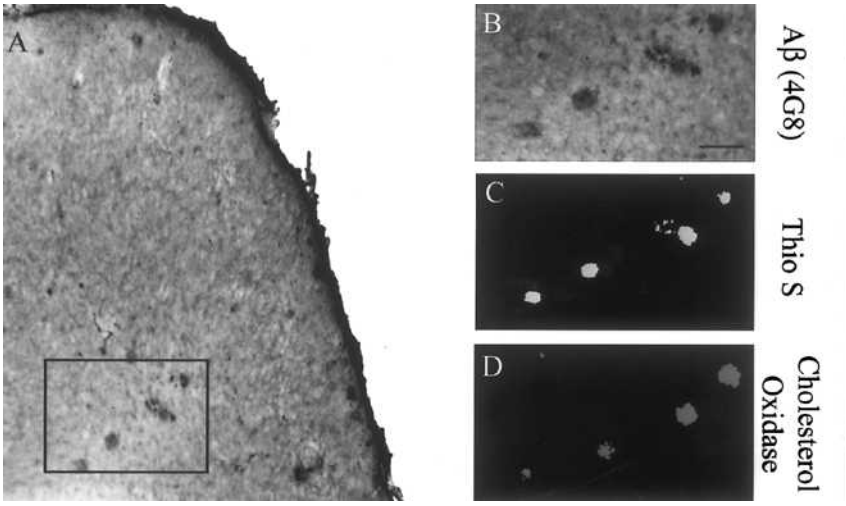

Figure 4. Co-localization of fibrillar amyloid with cholesterol oxidase in the cortex of a 12-month-old PS/APP mouse. (A) A low-power overview of amyloid plaques stained with 4G8. (B-D) Plaques from within the boxed areas double-labeled for Aβ (B) and thioflavine-S (C), and a consecutive section labeled for cholesterol oxidase (D). Scale bar = 100 um. Reprinted from Burns *et al.*, Co-localization of cholesterol, apolipoprotein E and fibrillar Aβ in amyloid plaques. *Molec. Brain Res.* **110:** 119-125, copyright (2003), with permission from Elsevier.

hypercholesterolemia is a risk factor in AD. In addition, differential expression of cholesterol hydroxylases has been advanced as a mechanism to explain the pathophysiology of AD (Brown *et al.*, 2004).

2.8 LM and *in vitro* Aβ fibrillogenesis

In their review on the structure of amyloid fibrils, Gorman and Chakrabartty (2001) show elegantly how LM usefully provides a basis from which the more molecular approaches used to study Aβ fibrillogenesis extend. Indeed, by direct observation the growth of amyloid fibrils can be monitored light microscopically by thioflavine-T fluorescence (Ban *et al.*, 2003). These workers used total internal reflection fluorescence microscopy (TIRFM) in a study of fibril dynamics for several different amyloid fibrils and were able to determine the exact length of growing Aβ fibrils. That slightly acidic conditions, such as pH 5.8, promote fibril formation from the Aβ peptide was shown with fluorescein-conjugated Aβ1-42 (Su and Chang, 2001), using the protocol established by Chang and Su (2000). This work

correlates well with the suggestion from cellular studies that Aβ present in acidic (lysosomal) organelles in neurones or microglia may initially form neurotoxic fibrils more readily than Aβ in neutral subcellular compartments.

3. TRANSMISSION ELECTRON MICROSCOPY

3.1 Technical background

The various techniques available to prepare specimens for transmission electron microscopy (TEM) have enabled extensive studies to be performed on human post-mortem and animal brain samples, and also on cultured neurones, microglia, astrocytes and other cells used for AD studies. Quite separate from these tissue and cellular studies, TEM has contributed widely to knowledege of reconstituted Aβ and other amyloid fibre-forming systems *in vitro*. Brain and cell culture studies have generally utilized conventional thin sectioning of resin-embedded samples, but more recently the addition of pre- and post-embedding antibody labeling has increasingly made a useful contribution. This latter represents a higher resolution extension to immunofluorescence LM and often enables precise localization of colloidal gold or other label on intracellular or extracellular structures definable by TEM study.

Oligomerization and fibrillogenesis of the Aβ peptides (primarily Aβ1-40, 1-42, and fragments) has received considerable attention from electron microscopists, often using the negative staining technique and more recently by cryo-TEM of unstained frozen-hydrated specimens; *see* Harris (1997) and Harris and Scheffler (2002) for techniques. The more specialist approach of scanning transmission electron microscopy (STEM) has also made a contribution. This technique enables a direct measurement of the mass of macromolecules to be made and determination of the mass/unit length of fibrils (Antzutkin *et al.*, 2002)). Combination of TEM with one or more biophysical/biochemical techniques (X-ray fibre diffraction, electrospray ionization MS, CD, NMR and solid state NMR spectroscopy, fluorescence spectroscopy, dynamic light scattering, small angle neutron scattering, SDS-PAGE, analytical ultracentrifugation) often provides the strongest possible approach for the study of fibrillogenesis. However, when applied with care, experience and knowledge of the biological samples, TEM alone has great potential, as will be demonstrated below.

3.2 Thin sectioning TEM studies of brain

At the subcellular level, thin section TEM often supports and extends the data available from parallel LM studies. This is impressively shown in several of the publications from Jerzy Wegiel and his colleagues (Figure 5). The role of microglial cells in fibrillar plaque formation in the brains of man and transgenic APP_{SW} mice was shown by Wegiel *et al.*, 2000; 2001a. This interpretation was subsequently supported by the study of Sasaki *et al.* (2002) using the Tg2576 transgenic mouse. Further evidence that vascular inflammatory microglial infiltration in transgenic mice contributes to the formation of cored Aβ plaques also comes for the ultrastructural study of Wegiel *et al.* (2003), using computer-aided 3D reconstruction of the tissue to assist the image interpretation. The LM study on hippocampal vascular fibrosis and calcification on normal aging, AD and DS subjects (Wegiel *et al.*, 2002) was supplemented by TEM, which strengthens the link between brain vascular pathology and sclerosis in AD.

Ultrastructural and immunocytochemical studies on neurones have been usefully used to assess the synaptic location and quantification of APP and presenilins in rodent brains (Ribaut-Barassin *et al.*, 2003). Understanding the change in synaptic distribution of these proteins during development and aging may be of use for the understanding of synaptic loss that precedes the deposition of extracellular Aβ. Supplementary to this, Takahashi *et al.* (2004) showed by immunoelectron microscopy that oligomerized Aβ accumulates within the neuronal processes and synapses of Tg2576 transgenic mice.

Using the transgenic *C. elegans* Alzheimer model Link *et al.* (2001) extended their extensive LM observations by immunogold TEM labeling. They provided convincing evidence for the location of extracellular Aβ deposits, which at high magnifications clearly showed a fibrillar ultrastructure. Simlarly, Verbeek *et al.* (2002) using cultured human brain pericytes, also showed multivesicle-like bodies by thin section TEM, with accumulation of APP.

Furthermore, the intracellular accumulation of Aβ within multivesicle bodies in neurones of normal human, rat and mouse brain was shown by Takahashi *et al.* (2002). For Tg 2576 mice and human AD brain the Aβ accumulation in microvesicles was more pronounced and increased with aging. This intraneuronal deposition of Aβ initially occurred with normal synaptic morphology and prior to extracellular plaque formation, thereby suggestive of a crucial role in the development of AD. In their TAS10 transgenic mouse AD model Richardson *et al.* (2003) also showed dystrophic neurons containing multi-vesicle/multi-lamellar inclusions and activated microglia, alongside amyloid plaque.

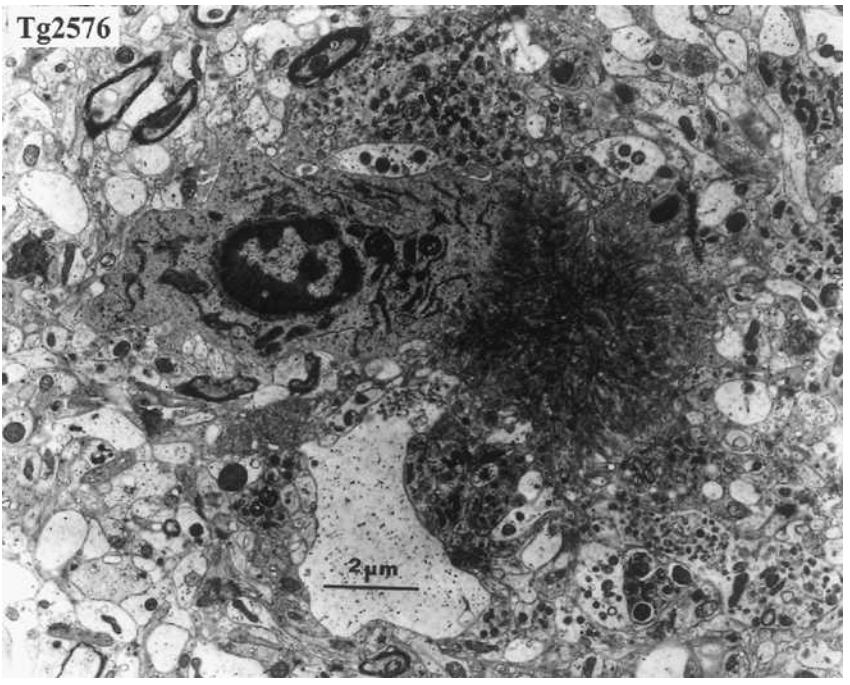

Figure 5. A small plaque in the thin sectioned brain of transgenic mouse (Tg2576). The micrograph show the body of a microglial cell, an immunolabeled amyloid star, dystrophic neurities and a few astrocytic processes. Previously unpublished micrograph, courtesy of Jerzy Wegiel.

Although there is evidence that Congo red and thioflavine-T (Th-T) inhibit cerebral Aβ fibrillogenesis, both these drugs fail to pass the blood-brain barrier (BBB). Use of polymeric nanoparticles as drug carriers, which have potential to pass the BBB has been investigated by Härtig *et al.* (2003). Fluorescent Th-T incorporated into latex nanoparticles was injected into the hippocampus of mice. After the photoconversion of Th-T it was detectable by LM and TEM, following injestion by microglia and subsequent aggregation. These authors speculated that such nanoparticles might be useful delivery system to target both intracellular and extracellular Aβ in the brain.

3D-Reconstruction from electron micrographs of amyloid deposits and microglial cells in the APP232 transgenic mouse model (Stalder *et al.,* 2001) conclusively showed that microglia do not phagocytose extracellular amyloid deposits and that there is no intracellular amyloid production in microglia. TEM immunogold-labelling of amyloid plaques and associated

inflammation, in PS2APP transgenic mice, was impressively correlated with progressive age-dependent cognitive deficits (Richards *et al.*, 2003). Using confocal LM and TEM, Hu *et al.* (2003) showed preferential infiltration of dystrophic cholinergic presynaptic boutons into fibrillar amyloid aggregates, in doubly (APP + PSI) transgenic mice. The implication of this observation was claimed to relate to the degeneration of cholinergic nerve terminals in advance of those of other neurotransmitter systems.

3.3 Amyloid-β oligomerization and fibrillogenesis

Despite some considerable overlap within the literature, it is appropriate to present this topic in three sections, dealing in turn with Aβ oligomers, protofilaments and fibrils.

3.3.1 Aβ Oligomers

Possible variability of the commerically available synthetic Aβ peptides was commented upon by Soto *et al.* (1995), with respect to their ability to form oligomers, protofilaments and fibrils. This was expressed as a varying ability to transform from α-helix to β-sheet. Since that time, reasonable standardization appears to have been achieved, yet data produced by different groups performing *in vitro* fibrillogenesis is not always in accord or mutually supportive.

A careful comparative analysis of oligomerization of the Aβ40 and Aβ42 peptides was performed by Bitan *et al.* (2003). They showed by SDS-PAGE that cross-linked oligomers revealed distinct small molecular mass distributions, with Aβ42 forming somewhat higher mass oligomers than Aβ40. TEM analysis by negative staining showed the presence of ~5 to 20 nm spherical particles (termed paranuclei), which aggregated to form larger elongated oligomers and clusters. Essentially similar convincing TEM evidence was provided earlier by Nybo *et al.* (1999), using Aβ42. Immunodetection of neurotoxic oligomers by their epitope specificity, this epitope being absent on the Aβ peptide and fibrils, may prove to be diagnostically useful (El-Agnaf *et al.*, 2003; Kayed *et al.*, 2003). Also, it is likely that future immunoelectron microscopical studies using the anti-Aβ-oligomer antibody will have considerable potential.

An α-helix containing Aβ oligomer was defined by Kirkadze *et al.* (2001). By utilizing Aβ peptides of difference amino acid sequence they concluded that asparagine and histidine are important for the conversion to protofilaments. Support for this concept also comes from the study of Fezoui and Teplow (2002), in which they proposed that a partly unfolded Aβ intermediate, still containing α-helix, is involved in fibrillogenesis.

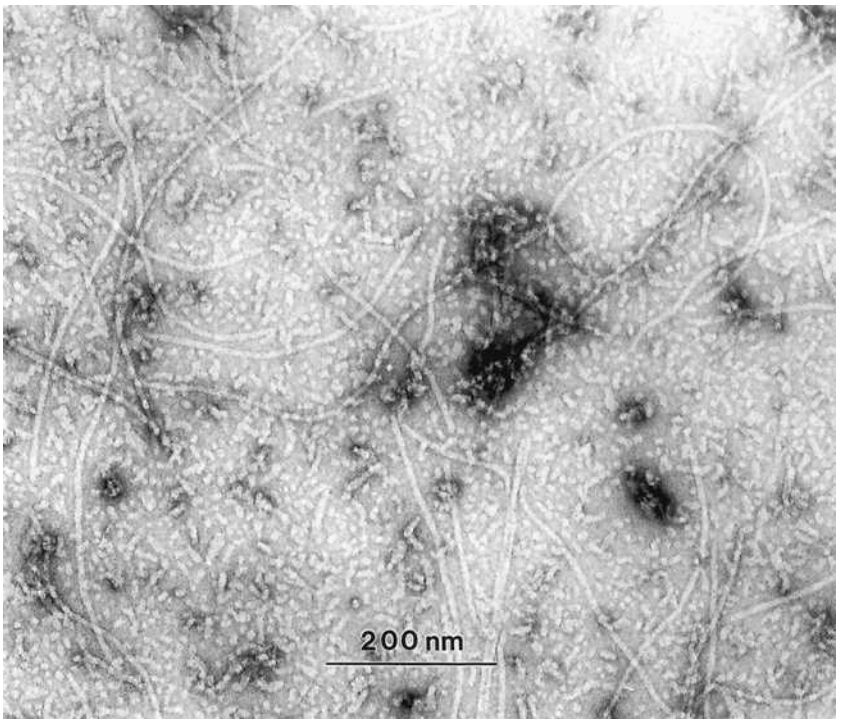

Figure 6. Oligomerization of human Aβ1-42 following incubation for 24h at 37°C in the presence of water alone. This negatively stained image shows that oligomers and flexuous protofibrils are present, but few mature fibrils (JRH, previously unpublished data).

Contrary to this, Huang *et al.* (2000) showed by CD spectroscopy that the very small Aβ oligomers contain an irregular secondary structure, i.e. no α-helix and no β-sheet, but that larger ~0.94 MDa oligomers do contain a β-sheet structure. This important observation may pin-point the Aβ oligomerization stage at which the α-helix to β-sheet conversion occurs, and thus enable the subsequent polymerization of a stable structural intermediate to proceed in order to generate the ~5 nm protofilaments and ~10 nm fibrils (*see below*). Although the TEM data discussed here does not provide any clear evidence that this intermediate (paranucleus) is a ring-like structure, others have produced averaged images of ring-like/annular oligomers or short protofilaments (Lashuel *et al.*, 2002a), which correlated with the concept that such oligomers could be responsible for penetration of the neuronal surface membrane. Our own data (Figure 6), using the human Aβ1-42 peptide shows that after 24 h incubation a range of short protofilament and smaller, possibly annular oligomers are present. From small angle

neutron scattering data Yong *et al.* (2002) concluded that a stable micelle-like oligomeric Aβ intermediate has a spherocylindrical shape (radius ~2.4 nm and height ~11 nm), but it is not clear that this relates directly to the annular oligomers of Lashuel *et al.* (2002).

Further evidence that Aβ oligomers can form ion channels comes for experimental work using lipid bilayers, liposomes, neurones, oocytes and other cells (Kagen *et al.*, 2002; Lin *et al.*, 2001); *see also* Chapter 17. The possibility that cell membrane cholesterol/ganglioside/sphingomyelin-rich microdomains (rafts) could be the predominant sites of Aβ interaction was advanced by Kakio *et al.* (2003).

A parallel is shown by the scrapie prion protein, which oligomerizes to form stable ~10 nm annular structures, that have the ability to form 2D crystalline arrays (Willie *et al.*, 2002). These prion annuli correlated with the interpretation that they stack or polymerize longitudinally to form the amyloid-like prion rods. Interestingly, a rapidly-formed but transient nano-crystalline from of a 14-amino acid Aβ peptide has been described by Otzen and Oliveberg (2004). Using TEM these workers showed that the nanocrystalline form of this peptide leads to the formation of a tangled aggregate (hours) and amyloid fibres (days).

3.3.2 Aβ protofilaments

Definition of the β-sheet-containing protofilament that can be formed by Aβ and several other fibril-forming amyloidogenic peptides is by no means clear and may differ from the putative annular oligomer described above. Indeed, Lashuel *et al.* (2000) considered that the protofilament is ~2-3 nm in width, representing a single linear crossed β-sheet assembly of the peptide, without any indication of a globular or annular repeating structure. It is likely that the different amyloidogenic peptides may not behave in an identical manner when they undergo the folding change necessary to generate 5-6 nm single or 10-12 nm double helical filaments and in some cases large multi-filament ribbons.

In a series of publications, many including TEM studies, David Teplow and his colleagues have defined the Aβ protofilament intermediates involved in fibrillogenesis (Walsh *et al.*, 1997; 1999). Again a combination of biophysical techniques was used, including size-exclusion chromatography to isolate the Aβ polymers of increasing mass. Their sequential model for fibrillogenesis is essentially similar to that of Lashuel *et al.* (2000), although a globular 'nucleus' or monomer aggregate (equivalent to the 'paranucleus' of Bitan *et al.*, 2003) is included prior to protofilament assembly.

In a recent study Lashuel *et al.* (2003), using the Arctic Aβ1-40 variant (E22G) as well as wild-type Aβ1-40, from extensive negative stain TEM

studies concluded that the pathogenic E22G variant more readily produced transformation of protofibrils to fibrils than did a mixture of E22G and wild-type.

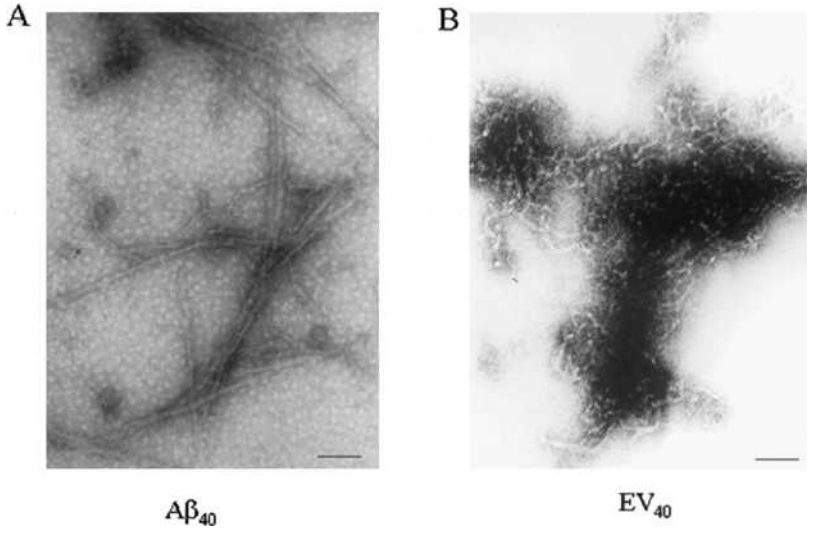

Figure 7. Negatively stained images of A1-40 (A) and EV40 (B) aggregates following incubation for 48h. Reprinted, with permission, from Qahwash *et al.*, Identification of a mutant amyloid peptide that predominantly forms neurotoxic protofibrillar aggregates. *J. Biol. Chem.* **278:** 23187-23195, © (2003).

Using a synthetic Aβ1-40, termed EV40, that had substitutions in the first two amino acids, Quhawash *et al.* (2003) showed by TEM that this variant could only form protofilaments, rather than fully-formed/mature Aβ fibrils (Figure 7). Remarkably, the EV40 peptide was even more toxic than Aβ1-40 towards neuroblastoma cells, and cortical and hippocampal neurones, suggesting the possible importance of this EV40 intermediate, beit protofilament or oligomer.

3.3.3 Aβ fibrils

In vitro conversion of clearly defined ~5 nm Aβ protofilaments to mature ~10 nm fibrils has not been intensively studied. It is acknowledged that some variability or fibril polymorphism exists for the different Aβ peptides, as for other fibril-forming peptides. In general, the mature Aβ fibril appears to be straight, with a smooth surface and a clear helical periodicity, generated from two protofilaments each of which itself contains two linear strands of helical crossed β-sheets. That this amyloid fibril then resembles a hollow

tubule was advanced by Malinchik *et al.* (1998), from X-ray diffraction and TEM data (negative stain and thin section). A representative example of mature helical Aβ1-42 fibrils produced by incubation in the presence of 1 mM hydrogen peroxide is given in Figure 8. Aggregation of Aβ1-40 fibrils to form large ball-like aggregates was shown by Westlind-Danielsson and Arnerup (2001). This distinguished the polymorphic behaviour of Aβ1-40 from Aβ1-42, which formed parallel fibril bundles.

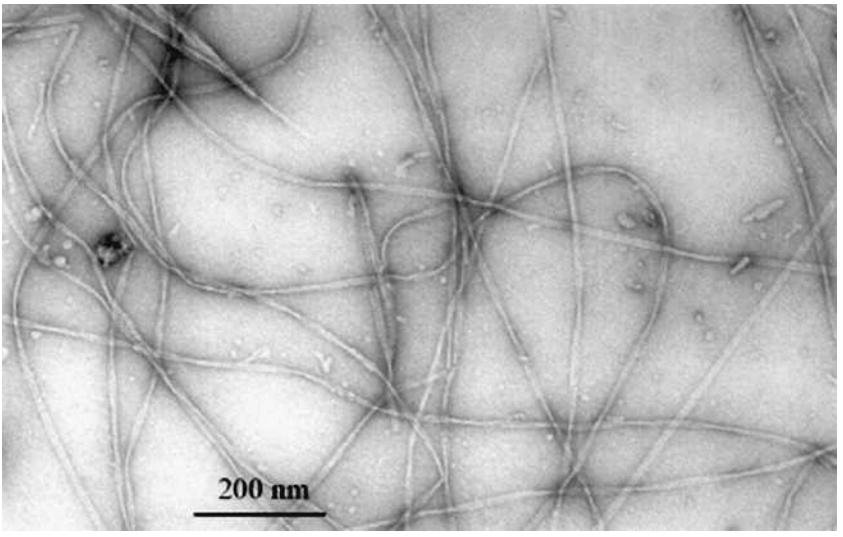

Figure 8. Amyloid-β1-42 fibrils formed after 24h 37°C incubation of the peptide (rat) in water in the presence of 1mM hydrogen peroxide (JRH, previously unpublished data).

The influence of pH on the fibrillogenesis of Aβ1-40 was assessed by Wood *et al.* (1996) using a range of biophysical techniques, including TEM. They concluded that at pH 5.8 Aβ1-40 tended to form turbid aggregates but at pH 7.4 transparent fibrils were formed. However, Abe *et al.* (2002), using the Aβ12-24 fragment found that 10-12 nm fibrils and 17-20 nm ribbons were formed at low pH. Others have attempted to define which amino acid residues are important for fibril formation (Fraser *et al.*, 1994; Bond *et al.*, 2003; Tjernberg *et al.*, 2002). The role of Aβ1-42 Gly33 and Meth35 in relation to neurotoxicity and free radical attack was advanced by Kanski *et al.* (2001). These workers also implicated the hydrophobic environment of Meth35 in the neurotoxicity and oxidative properties of Aβ1-42 (Kanski *et al.*, 2002). TEM and STEM were combined with solid state NMR to assess the fibrillogenesis of Aβ10-35, Aβ1-40 and Aβ1-42 by Antzutkin *et al.*

(2002). The value of mass/unit length measurement by STEM in relation to determination of the number of single filament crossed-β-sheets and protofilaments present in any one mature fibril was clearly demonstrated.

Using negative stain TEM as one of their approaches, Romano *et al.* (2003) assessed the structure of both synthetic and neuronally secreted Aβ fibrils. Both types of fibrils labeled with monocolonal antibodies against Aβ amino acid epitopes 1-17 and 17-24, as shown by immunonegative staining. Significantly, however, only the naturally secreted neuronal fibrils interfered with long-term memory loss in a crab learning model. Definition of the aa sequence of Aβ responsible for fibril formation has been investigated by Williams *et al.* (2004). By scanning proline mutagenasis they shwoed that the Aβ15-36 β-sheet-rich core region could create a hydrogen-bonded stack of peptides. Again, negative stain EM contributed to the understanding of the structure of the different proline mutant Aβ fibrils.

Further TEM and STEM work on short synthetic Aβ fragments of known length and sequence, as well as on the naturally occurring mutant Aβ peptides, is likely to continue to provide useful data.

3.3.4 Aβ fibril-associated proteins

From biochemical microplate-binding studies Hamazaki (1995) showed a calcium-dependent affinity of Aβ1-40 for serum amyloid P component (SAP). This has been confirmed using TEM by others, including the author, and appears to be of significance for the inflammatory response and the coating of Aβ fibrils with SAP may also prevent or reduce the natural proteolytic degradation of Aβ deposits. Nielsen *et al.* (2000) have shown by TEM of negatively stained specimens that SAP and heparan sulphate proteoglycan both bind to pre-formed fibrils. Binding was confirmed by immunostaining with 5 nm Protein A/G-gold particles after on-grid incubation with polyclonal anti-SAP. Our own data essentially agrees with that of Holm Nielsen *et al.* (2000), with the additional observation that mature Aβ fibrils bind SAP more efficiently than protofibrils (Figure 9). This may correlate with the varying binding of SAP in diffuse and dense cored amyloid plaques.

A link between SAP complement C1q and microglial activation was advanced by Veerhuis *et al.* (2003), using LM, TEM and ELISA assays. A marked increase in Aβ1-42 fibril formation was claimed in the presence of C1q and SAP. This implies that the inflammatory response may actually potentiate Aβ fibrillogenesis. That α1-antichymotrypsin regulates Aβ fibrillogenesis was shown by Eriksson *et al.* (1995) and the cysteine protease inhibitor cystatin C has also been localized in Aβ deposits (Vattemi *et al.*, 2003).

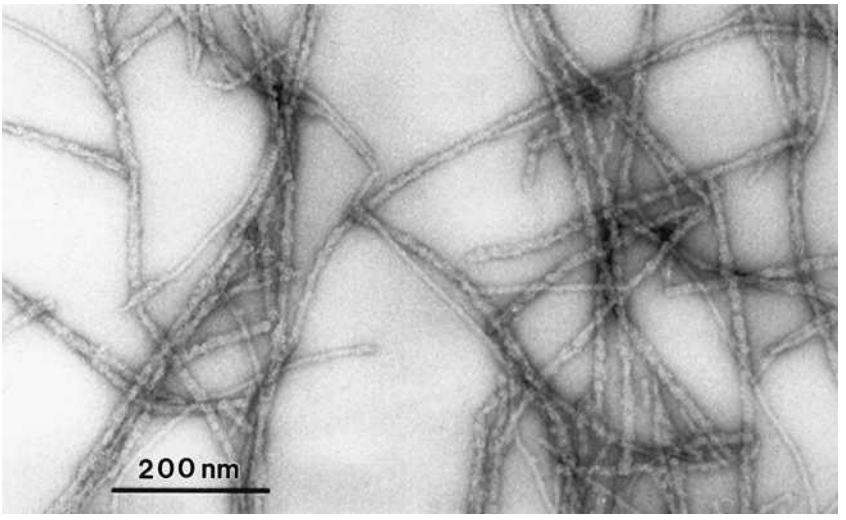

Figure 9. Amyloid-β1-42 fibrils (human aa sequence) formed in the presence of 0.5 mM H_2O_2 with bound SAP (+ 0.5 mM calcium and magnesium chloride). Note the coating of the mature paired helical fibrils by SAP, but the absence of binding to the single protofibrils (JRH, previously unpublished data).

The biochemical evidence for catalase binding to Aβ peptide and fibrils (*see also* section **2.6**) has recently been supported by TEM studies, as shown in Figure 10. Although the amino acid sequence of Aβ responsible for the affinity of catalase has been defined, it is not clear how this might influence fibrillogenesis, other than by inhibition of the anti-oxidant activity of this enzyme.

Acetylcholinesterase (AChE) has been shown to accelerate Aβ fibrillogenesis (Di Ferrari *et al.*, 2000; Inestrosa *et al.*, 1996). In both these publications TEM evidence was presented for binding of AchE to Aβ fibrils (Figure 11). Inhibition of AchE and AchE-induced Aβ fibrillogenesis by the drug 3-(4-{[benzyl(methyl)amino]methyl}-phenyl)-6,7-dimethoxy-2H-2-chromenone (AP2238) (Piazzi *et al.*, 2003), designed initially to bind to both the catalytic and peripheral sites of AchE, indicates the possible value of AP2238 for AD therapy.

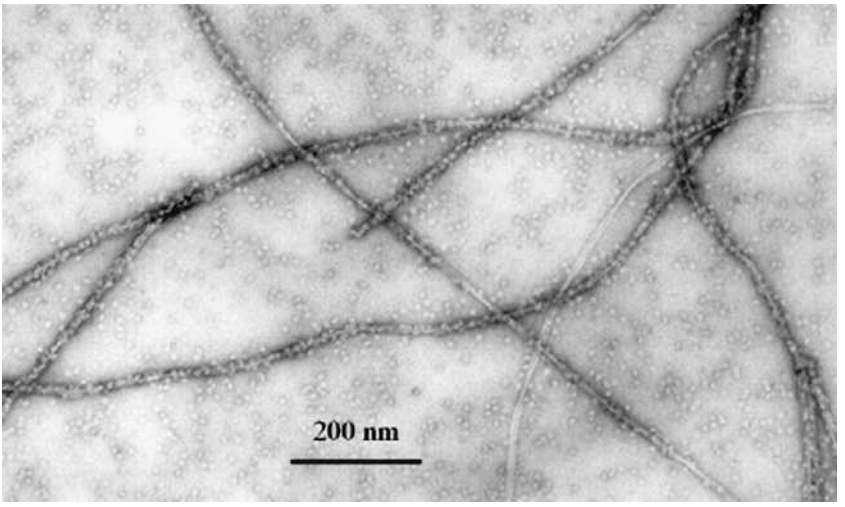

Figure 10. Amyloid-β1-42 fibrils (human aa sequence) formed in the presence of 0.5 mM H_2O_2, with bound human erythrocyte catalase (JRH, previously unpublished data).

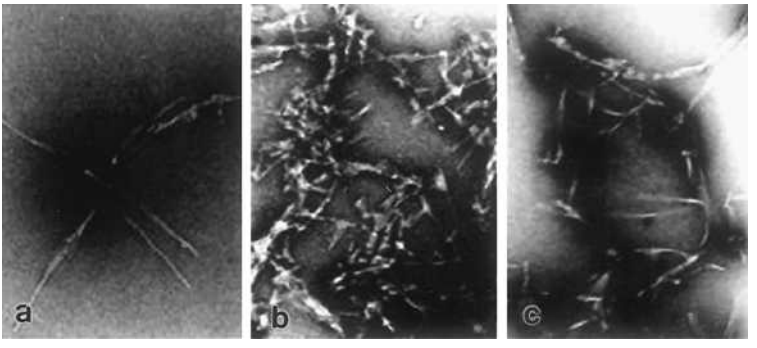

Figure 11. Amyloid –β fibrils (human aa sequence) with bound acetylcholinesterase. (a) Aβ1-40 fibrils alone. (b) Aβ fibrils assembled in the presence of *Torpedo* AChE. (c) Aβ fibrils assembled in the presence of the hydrophobic AChE fragment $H_{274-308}$. Reprinted with permission from Di Ferrari *et al.*, A structural motif of acetylcholinesterase that promotes amyloid β-peptide fibril formation. *Biochemistry* 40: 10447-10457. Copyright (2001) American Chemical Society.

The LM study of Goldstein *et al.* (2003) on αB-crystalin (see also section **2.6**) was supplemented by successful TEM double immunogold labeling of Aβ protofilament/fibril aggregates with bound αB-crystalin. Binding of Aβ by mitochondrial alcohol dehydrogenase (Lustbader *et al.*, 2004) has been linked to mitochondrial dysfunction and neuronal oxidative stress in AD.

3.3.5 Aβ fibrillogenesis and cholesterol

Despite the considerable LM and biochemical evidence for cholesterol localization in Aβ deposits in brain, the available supportive evidence from TEM is limited. This may be because of the difficulty encountered when attempting to stain for cholesterol in thin sectioned tissue and the lack of knowledge regarding the chemical state (molecular, micellar or microcrystalline) of any cholesterol that is bound to or associated with Aβ fibrils, and the esterification state of any such cholesterol. However, direct addition of cholesterol microcrystals or micellar "soluble cholesterol" (polyoxyethyl cholesterylsebacate) to Aβ1-42 peptide at the start of the fibrillogenesis incubation has been found to potentiate fibril formation (Harris, 2002), and provided evidence for cholesterol binding/association with mature fibrils (Figure 12). Addition of these two cholesterol reagents to pre-formed Aβ fibrils has also shows the binding of Aβ fibrils to cholesterol. Note that this affinity of Aβ for cholesterol is present in the absence of the cholesterol-binding lipoprotein $ApoE_4$, which has itself an affinity for Aβ.

McLaurin *et al.* (2000) assessed the action of several proteins and lipids on the secondary structural characteristics of Aβ and on Aβ fibrillogenesis. They provided strong supportive evidence that interaction of chondriotin and heparan sulphate proteoglycans enhances transformation to crossed β-sheet, with fibril formation. Among the phospholipids they investigated, both phosphatidylserine and phosphatidylinositol enhanced Aβ fibril formation, as did iron, copper and zinc ions; however, the potentiating effects of cholesterol and hydrogen peroxide was not included in this study. The involvement of heparan sulphate proteoglycans in AD has been reviewed by van Horssen *et al.* (2003), with emphasis upon LM and biochemical aspects.

3.3.6 Drug inhibition of Aβ fibrillogenesis

Numerous compounds have been claimed to retard or inhibit Aβ fibrillogenesis. Often studies rely upon reduction of Congo red or thioflavine-T (Th-t) fluorescence, as a measure of reduced oligomerization

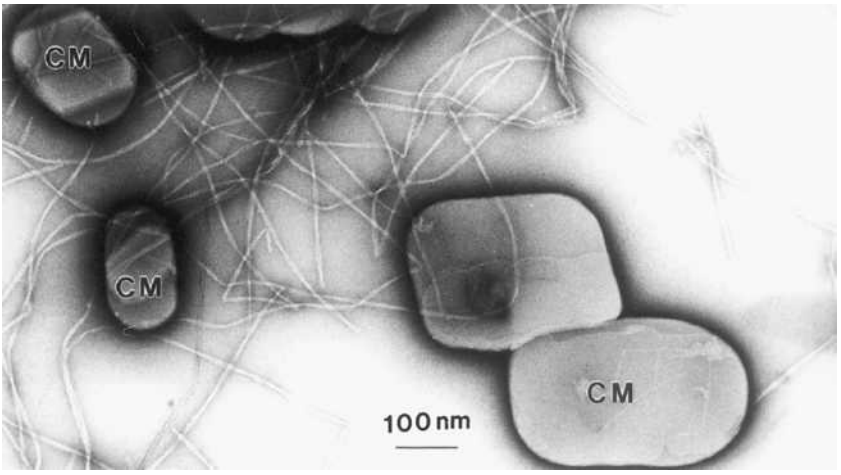

Figure 12. Amyloid-β1-42 fibrils (rat) assembled in the presence of cholesterol microcrystals (CM). Modified from Harris (2002).

and fibril formation; only work relating to or including TEM assessment will be mentioned here (for LM, see section **2.8)**. Indeed, an inhibitory action of Congo red itself was found by Lorenzo and Yankner (1994) when this compound was added to the Aβ peptide at the start of *in vitro* fibrillogenesis. The hormone melatonin has been shown by Pappola *et al.* (1998) to inhibit Aβ fibrillogenesis, but the related compound 5-hydroxy-N-acetyl-tryptane had no effect, neither did the powerful non-specific anti-oxidant N-t-butyl-α-phenylnitrone.

In Switzerland, the EM groups of Jacques Dubochet and Ueli Aebi have independently both been involved in significant investigations on inhibition of Aβ fibril assembly, in collaboration with pharmaceutical companies. Goldberg *et al.* (2000) provided both TEM and STEM data that enabled characterization of the polymorphic Aβ1-40 fibril assemblies to be assessed. From their extensive data a scheme advanced for the development of fibril polymorphism was advanced, which is essentially similar to that of Lashuel *et al.* (2000). The inhibitory interaction of several morpholinotriazines was considered by Bohrmann *et al.* (2000), from their TEM and biophysical data, to occur at the level of small Aβ1-42 structural intermediates.

Remarkably, Kiuchi *et al.* (2002a) maintained that type IV collagen inhibits Aβ1-40 fibrillogenesis, and Ueda *et al.* (2002) showed that high concentrations of sucrose (1.5 M) retarded the fibrillogenesis of Aβ1-42. Significantly, in the presence of sucrose, a compact amorphous Aβ1-42 aggregate remained which did not produce Th-T fluorescence (*cf* the effect

of aspirin, below). The effect on Aβ1-40 fibrillogenesis of a new class of apomorphine derivatives was investigated by Lashuel *et al.* (2002), using TEM, Th-T fluorescence and analytical ultracentrifugation. Auto-oxidation products of the drugs apparently acted directly on the Aβ peptide to inhibit fibrillogenesis. That the commonly used analgesic and anti-inflammatory drug aspirin, and other non-sterol anti-inflammatory drugs (NSAIs) such as probucol, have potential as inhibitors of Aβ fibrillogenesis has been indicated in the clinical literature for a number of years. Direct *in vitro* evidence that aspirin inhibits Aβ1-42 fibrillogenesis comes from the author's own TEM work (Harris, 2002), as shown in Figure 13.

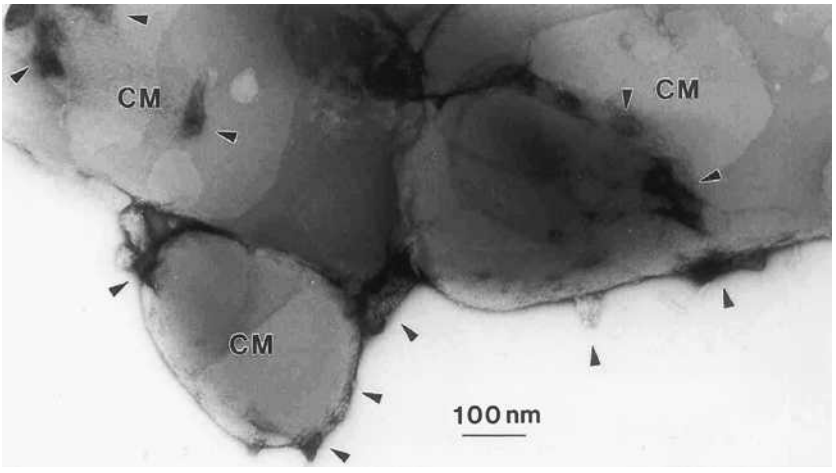

Figure 13. The inhibition of cholesterol-potentiated Aβ1-42 (rat) fibrillogenesis by 1 mM aspirin. Note the absence of fibrils and the presence of small Aβ clusters (arrowheads) binding to the cholesterol microcrystals (CM). Modified from Harris (2002).

Because of the therapeutic potential, in addition to basic understanding of the inhibition of Aβ fibrillogenesis, considerable interest has also be directed towards the possibility that drugs and other compounds may produce disassembly of pre-formed Aβ fibrils, detectable by TEM and correlated with reduction of Th-T fluorescence. In our own studies, only a slow and possibly insignificant effect was detected with aspirin and other compounds require further careful investigation using pre-formed mature Aβ fibrils prepared in the presence of cholesterol or low concentrations of hydrogen peroxide.

Basement membrane components (collagen IV, entactin and liminin) have been shown to be reactive by producing disassembly of pre-formed Aβ1-42 (Kiuchi *et al.* 2002b). This correlates with the work of Kiuchi *et al.*

(2002a), mentioned above, where they inhibited fibrillogenesis using collagen IV alone. Similarly, the anti-oxidant nordihydroguaiaretic acid can apparently both inhibit Aβ fibrillogenesis and produce breakDown's of pre-formed fibrils (Naiki *et al.* 1998; Ono *et al.*, 2002a). The same group also showed that nicotine prevents Aβ fibrillogenesis and induced fibril breakDown's (Ono *et al.*, 2002b), due specifically to the N-methylpyrrolidine moiety. If this is the case in man, it would seem possible that nicotine could readily be administered to Alzheimer patients orally or transdermally, but addiction is likely and other side effects would need to be carefully monitored. An alternative explanation for the action of nicotine has been advanced by Dickerson and Janda (2003). These workers linked Aβ glycation as the likely mechanism for the neuroprotection by nicotine. That both the D-(+) and L-(-) enantiomers of nicotine interfere with Aβ fibrillogeneis and cytotoxicity was shown by Moore *et al.* (2004). A more disrupted type of Aβ aggregate, shown by negative staining, was formed in the presence of nicotine, rather than well defined fibrils. The reduction of insoluble Aβ deposits in the brain of APPsw transgenic mice by relatively short-term (10 day) administration of nicotine (Hellstrom-Lindhal *et al.*, 2004) implies a direct action of this drug on amyloid fibres and an influence on their physiological clearance.

4. ATOMIC FORCE MICROSCOPY

4.1 Technical background

When the scanning probe microscopies (SPMs), atomic force microscopy (AFM) and scanning tunneling microscopy (STM), were first applied to biological samples the images produced were often difficult to interpret and fraught with artefacts. Today, this situation has largely been overcome. Confidence can be placed in the validity of the images, especially due to correlative studies where both TEM and SPM have been used, as is often the case within the Alzheimer and other fields of study. It is also apparent that the quality of SPM images has improved significantly throughout the past ~10 years, and new modes of imaging have extended the possibilities for the high resolution study of biological structures. For technical detail the reader is referred to the recent review Yang *et al.* (2003). As with the TEM studies on Alzheimer fibrils, SPM studies are often linked to LM and the various biophysical approaches, in particular light scattering, the spectroscopies, analytical ultracentrifugation and SDS-PAGE.

The first SPM contributions to Aβ fibrillogenesis appeared in the mid-1990s. Since then there has been a marked expansion of the available data,

particularly from AFM. Although there is considerable overlap and agreement between the data from AFM and TEM, only the former has so far provided useful information on Aβ oligomerization dynamics and the formation of protofilaments; both have provided data on the oligomerization and the structure and polymorphism of mature Aβ fibrils.

Instead of subdividing the specific contributions from AFM and STM in the manner of section **3** above, it is more appropriate to now summarize the available SPM data as a whole. An element of chronological order will be included, with indication of the larger groups that have made major contributions and the smaller groups whose contributions may be no less significant.

4.2 AFM: Aβ oligomerization and fibrillogenesis

One of the first assessments of progressive Aβ1-40 filament assembly came from Shivji *et al.* (1995), using STM with platinum-carbon-coated samples. The AFM was used by Oda *et al.* (1995) to show inhibition of Aβ1-42 fibril formation by clusterin (ApoJ) (*see also* Chapter 14). Smaller, more slowly sedimenting Aβ complexes were produced in the presence of clusterin, which induced greater oxidative stress and neurotoxicity, compared to untreated Aβ fibrils. Elongation of Aβ1-40 protofilaments by addition of monomers (and oligomers) and the lateral association of protofilaments was monitored by Nichols *et al.* (2002) using light scattering and AFM.

In a series of articles Peter Lansbury and colleagues have presented AFM data on comparative fibrillogenesis, with emphasis on the formation and structure of Aβ1-40 protofilaments (Harper *et al.*, 1997a,b: Harper *et al.*, 1999; Koo *et al.*, 1999). More recently, JoAnne McLaurin and colleagues have published extensive AFM data demonstrating the structure of soluble oligomers of Aβ1-40 (Huang *et al.*, 2000) and the interaction of Aβ1-28, Aβ1-40 and Aβ1-42 with lipid bilayers (Yip and McLaurin, 2001, Yip *et al.*, 2002). Their review on modulating factors and Aβ fibrillogenesis (McLaurin *et al.*, 2000) remains a valuable contibution to the subject that presents both TEM and AFM data. The incorporation of both Aβ1-40 and Aβ1-42 into mixed pre-fibrillar aggregates was assessed by fluorescence, CD spectroscopy and AFM (Frost *et al.*, 2003). This approach is important in view of the varying deposition of Aβ1-40 and Aβ1-42 in diffuse and senile plaques.

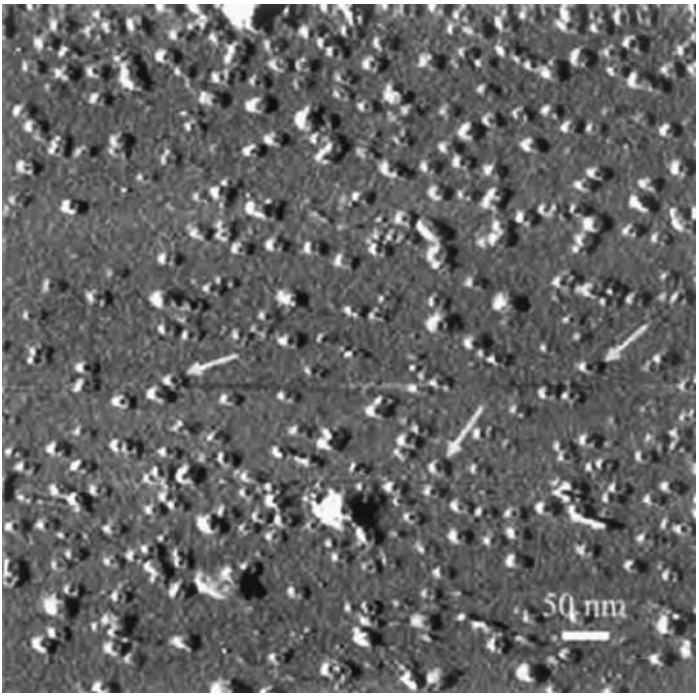

Figure 14. An AFM image Amyloid–β1-42 reconstituted on a planar bilayer membrane. Individual donut-shaped structures protruding from the membrane surface represent individual channels (arrows). Reprinted, with permission, from Lin *et al.*, Amyloid β protein forms ion channels: implications for Alzheimer's disease pathophysiology. *FASEB J.* 15: 2433-2444. Copyright (2001).

By spreading Aβ1-42 for AFM on the surface of mica (hydrophilic) and graphite (hydrophobic) Kowalewsk and Holtzman (1999) showed structural differences in Aβ assembly. On graphite, sheet-like parallel linear aggregates were formed, whereas on mica Aβ globules and short globular protofibril chains were produced. These workers suggested that their observations could have relevance for Aβ interaction with the hydrophobic membrane bilayer and lipoproteins. More recently, the same group (Legleiter *et al.*, 2004), assessed the effect of two sequence-specific anti-Aβ monocolonal antibodies on Aβ fibrillogenesis by AFM. An antibody against the N-terminal aa residues 1-5 slowed fibril formation, whereas an antibody against aa residues 13-28 prevented fibril formation. Liu *et al.* (2004a) using ThT fluorescence and AFM showed that single chaing variabale antibody fragment (scFv) to aas 17-28 of Aβ inhibited both Aβ aggregation and neurotoxicity. Aβ aggregation on hydrophobic graphite was also studied by high resolution AFM and STM by Wang *et al.* (2003), who suggested that the Aβ fibrils had

a characteristic right-handed helical twist with an underlying domain texture due to association of protofilaments and monomers.

Lin *et al.* (2001) who showed by AFM that globular oligomers of Aβ1-42 formed in solution at short incubation times, but when the peptide was incorporated into planar lipid bilayers 8-12 nm diameter ring-like ion channels were formed (Figure 14). These workers also correlated their data with cellular studies using cultured neurones and LM. Changes in neurone morphology and viability were assessed following the addition of Aβ1-42, to emphasize the likely significance of the ion channels with respect to production of neuronal death.

AFM has considerable potential for dynamic "real-time" aqueous imaging of Aβ oligomerization and fibril growth, as demonstrated by Goldsbury *et al.* (2001) and Parbhu *et al.* (2002). By TEM, samples can readily be made at increasing time intervals from the start of Aβ incubation, but by AFM the growth of the same individual fibril can be monitored over a period of time (Figure 15).

An impressive combination of AFM with SDS-PAGE, determination of oligomer mass by ultracentrifugation and neuronal viability testing has recently been published by Dahlgren *et al.* (2002) and Stine *et al.* (2003). Particular care was taken by these workers to standardize their oligomerization and fibrillogenesis protocols, with Aβ1-40, Aβ1-42, and the Dutch and Arctic mutants of Aβ1-42. Attention to such detailed aspects is of undoubted importance for the accurate structural definition of the small and larger Aβ oligomeric intermediates and the correlation of oligomer structure and fibrillogenesis with neurotoxicity.

Fibril-free stable neurotoxic globular Aβ1-42 oligomers, produced at 4°C by Chromy *et al.* (2003), were analysed in detail by AFM and non-denaturing gel electropheresis. Furthermore, these workers showed that neuroprotection by *Ginkgo biloba* extracts correlated with inhibition of Aβ oligomer formation. By co-incubation of Aβ1-40 with the fragments Aβ25-35 and Aβ17-40 Liu *et al.* (2004b) concluded from AFM studies that the residues 17-20 and 30-35 play a critical role in the formation of oligomers and protofibrils.

5. CONCLUDING COMMENTS

The diversity of the microscopical data presented above indicates the many exciting aspects of current research into amyloid-β peptide oligomerization and polymerization to form fibrils and Aβ involvement in the pathogenesis of Alzheimer's disease. Nevertheless, some may readily dismiss the contribution of microscopy; this chapter attempts to rectify this

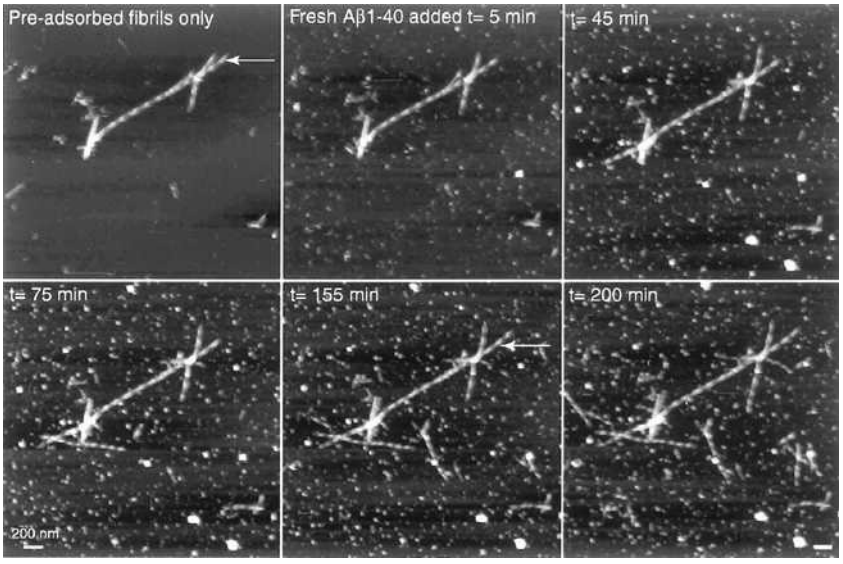

Figure 15. Seeded growth of Aβ1-40 fibrils on a mica surface, imaged by AFM. Reprinted from Goldsbury *et al.*, Visualizing the growth of Alzheimer's Aβ amyloid-like fibrils. *TRENDS in Molec. Med.* 7: 582. Copyright (2001), with permission from Elsevier.

misguided opinion. LM, TEM and AFM all continue to make a major contribution to the Alzheimer field. This is best exemplified by quoting the most recent publications where microscopy makes a major contribution (*e.g.*, Anderson *et al.*, 2004; Li *et al.*, 2003; Miu and Andreescu, 2003; Ribaut-Barassin *et al.*, 2003; Richardson *et al.*, 2003; Schwab *et al.*, 2004). Indeed, the combination of several microscopical techniques, alongside NMR and electron paramagnetic resonance, was reviewed by Antzutkin (2004), for the study of Aβ fibrillogenesis and metal binding by amyloid fibrils.

Although the modern microscopies can be stand-alone techniques, their strength is best shown when the data they produce is integrated with that from cellular, biochemical and biophysical approaches. This important aspect is not neglected above. There is little doubt that the combination of immunology with microscopy, as demonstrated by the use of monospecific polyclonal and monoclonal antibodies as labels for subcellular structures and for molecular-level labeling of Aβ oligomers, protofilaments and fibrils *in vitro*, and the other amyloid plaque-associated proteins, has even more to contribute.

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