**Title:** The complexities underlying Age-related macular degeneration- could Amyloid beta play an important role?

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**Abstract:**

Age-related Macular Degeneration (AMD) causes irreversible loss of central vision for which there is no effective treatment. Incipient pathology is thought to occur in the retina for many years before AMD manifests from midlife onwards to affect a large proportion of the elderly. Although genetic as well as non-genetic/environmental risks are recognised, its complex aetiology makes it difficult to identify susceptibility, or indeed what type of AMD develops or how quickly it progresses in different individuals. Here we summarise the literature describing how the Alzheimer’s-linked Amyloid beta (A) group of misfolding proteins accumulate in the retina. The discovery of this key driver of Alzheimer’s disease in the senescent retina was unexpected and surprising, enabling an altogether different perspective of AMD. We argue that A fundamentally differs from other substances which accumulate in the ageing retina, and discuss our latest findings from a mouse model in which physiological amounts of A were subretinally-injected to recapitulate salient features of early AMD within a short period. Our discoveries as well as those of others suggest the pattern of A accumulation and pathology in donor aged/AMD tissues are closely reproduced in mice, including late-stage AMD phenotypes, which makes them highly attractive to study dynamic aspects of A-mediated retinopathy. Furthermore, we discuss our findings revealing how A behaves at single-cell resolution, and consider the long-term implications for neuroretinal function. We propose A as a key element in switching to a diseased retinal phenotype, which is now being used as a biomarker for late-stage AMD.

**Key words:** Amyloid beta (A), retinal neurons, retina, mouse models, Age related Macular Degeneration (AMD).

**Introduction**

Recent demographic shifts in many parts of the world have resulted in an increasingly ageing population. For many of the elderly however, prolonged lifespans are spent with chronic illnesses, reduced independence and in increased need of care. Chronic degenerative conditions such as Age-related Macular Degeneration (AMD) and Alzheimer’s disease (AD) have complex aetiologies and exhibit several disease phenotypes. Critically, these diseases have no effective treatments and impose a growing burden on limited public health resources (Hebert et al., 2003; Gordois et al., 2012). Here, we focus on AMD; a common blinding disease in which central vision becomes irreversibly lost (Lotery and Trump, 2007; Khandhadia et al., 2012). Hence, patients become unable to participate in routine activities such as recognising faces, reading and driving. AMD patients describe the impact of sight loss as a form of bereavement, whilst its long-term consequences include depression, reduced mobility and social isolation (Owen et al., 2003). The mammalian retina is arranged in such a manner that light-sensitive photoreceptors are found in the outer retina, hence light has to pass unimpeded through the intervening inner retina to reach the photoreceptor layer (Figure 1A). Immediately adjacent to photoreceptors and in intimate association is a polarised monolayer of cells referred to as the Retinal Pigment Epithelium (RPE). The RPE layer sits on a pentalaminar tissue referred to as Bruch’s membrane (BrM) which, together with the RPE, forms the blood-retinal-barrier (BRB) to create an immune-privileged ocular environment that separates the eye from the vasculature. The RPE however, also carries out a number of other functions that are critical to healthy retinal function and normal vision (Marmor and Wolfensberger, 1998). The outermost layer of BrM in-turn forms an integral part of the choriocapillaris; a dense network of blood vessels of the choroid providing oxygen/nutrients to the outer retina whilst removing metabolic waste (Bhutto and Lutty, 2012) (Figure 1A).

As the name implies, AMD is characterised by irreversible damage to the central region of the retina, termed the macula (Figure 1B), which provides focused vision. In most cases, primary pathology develops in the RPE, which has therefore been a major focus of research (Marmor and Wolfensberger, 1998; Bhutto and Lutty, 2012; Khandhadia et al., 2012). Incipient retinal pathology is thought to develop at cellular level for many years although patients do not initially report any visual problems. The development of protein/lipid aggregates at the RPE-BrM interface in the macula is considered to be the first clinical indicator of increased susceptibility (Sarks et al., 1999). Such deposits, termed drusen, appear as yellow-white spots when viewed through an ophthalmoscope (Figure 1C), although even at this stage patients remain largely asymptomatic (Bhutto and Lutty, 2012; Khandhadia et al., 2012). Early AMD was observed in 3.8% of individuals as young as 35-45 years of age (Korb et al., 2014), which rose to 9.8% in those aged 65 years or older (Klein et al., 2010). This early stage may then progress to intermediate and late AMD, where two broadly-defined phenotypes develop: geographic atrophy (GA) or vascular AMD (Lotery and Trump, 2007; Khandhadia et al., 2012). By the time individuals reach the 8th decade of life, the prevalence of sight threatening late-stage AMD has increased to ~12% (Friedman et al., 2004). Of the two late-stage phenotypes, GA AMD is defined by the development of a macular lesion, corresponding to an area in which RPE and photoreceptors have atrophied. As a consequence, the underlying choroid becomes more visible when viewed through an ophthalmoscope (Figure 1D). Vascular AMD is characterised by primary RPE pathology and a compromised BRB through which proliferative choroidal vessels invade the retinal environment. These leaky vessels release fluids or exudates which accumulate beneath the RPE/retina to cause retinal detachment, and form a fibrous scar over time (Figure 1E). Vascular AMD is routinely managed via intravitreal injections of anti-Vascular Endothelial Growth Factor (VEGF) inhibitors such as ranibizumab (Lucentis) and aflibercept (Eylea) or the off-label Avastin (Amoaku et al., 2015). Although patients report significant sight improvements, this strategy is largely unsatisfactory in the long term as initial visual gains are almost impossible to maintain, whilst some individuals do not respond to treatments at all. Critically, prolonged treatment appears to cause a switch in some patients to the GA form of AMD (Lois et al., 2013; Grunwald et al., 2015) which has no treatment at all. It appears that relative frequencies of GA and vascular AMD are broadly similar if only late disease is compared (Owen et al., 2012), which means that at present, approximately half of late AMD patients do not even have a strategy to manage the disease. This lack of meaningful AMD treatment bodes poorly for ageing populations throughout the world. For instance, AMD currently affects >½ million individuals in the United Kingdom alone, and is expected to increase to 680,000 cases by 2020 (source Macular Society, UK). Globally, early stages of AMD are estimated to affect >150 million individuals (Wong et al., 2014). As populations age, an increasing number of AMD cases are also being reported in rapidly developing countries (Krishnan et al., 2010; Ye et al., 2014; Elias et al., 2015) which add to existing high AMD numbers in the developed world. The disease is also aggressive, rapidly progressing from largely asymptomatic early stages to advanced AMD which is when sight loss occurs.

For instance, ~15.% of individuals with soft indistinct drusen or 20% of those with RPE abnormalities progressed to late-stage AMD within a 10 year period (Klein et al., 2002). This rate of disease progression is reflected in the high incidence of late-stage AMD patients, estimated to be approximately 71,000 newly diagnosed cases that are annually recorded in the United Kingdom (Owen et al., 2012). The high prevalence of advanced AMD cases is also evident worldwide, which is predicted to affect approximately 10 million individuals (Wong et al., 2014).

It is unclear whether the two different AMD phenotypes result from the consequences of a single disease affecting different tissues of the outer retina or is in fact a combination of several diseases. The conventional view has been that primary RPE impairment leads to subsequent photoreceptor damage and eventual sight loss (Lotery and Trump, 2007; Khandhadia et al., 2012). It must be noted however that the primary site of AMD pathology is not solely restricted to the RPE, but can also manifest in the photoreceptor layer, as well as the choroid (Spaide, 2009; Bird et al., 2014). For instance, in some cases photoreceptor atrophy is observed in the absence of any underlying RPE pathology (Bird et al., 2014). Differences in *where* disease first manifests make it difficult to study its aetiology, which is made even more challenging as AMD diverges to distinct GA and vascular phenotypes. To complicate matters further, in some instances AMD can also develop in the absence of any macular drusen. Such observations reinforce the idea that complex and ill-defined mechanisms may be at play which trigger and/or drive AMD at different speeds towards different outcomes in different individuals. The Age Related Eye Disease Study (AREDS) classifies and grades AMD to reflect these subtleties and to account for the diverse range of reported clinical phenotypes (Davis et al., 2005). Recently, the appearance of drusen between the RPE and photoreceptors, referred to as reticular pseudodrusen, was shown to indicate a greater risk of progressing to advanced AMD compared to eyes with drusen only (Spaide, 2013; Sivaprasad et al., 2016). However, classification is still largely based on clinical observations, which means that incipient cellular changes that occur before macular drusen becomes apparent may remain undetected. Recent evidence from patients treated with anti-VEGF inhibitors could potentially offer clues to the underlying pathology of AMD, as prolonged treatment can induce GA. The mechanisms by which RPE damage occurs under these circumstances are still unclear, although a putative correlation was observed between increased numbers of anti-VEGF injections and the severity of RPE damage (Lois et al., 2013). One suggestion is that this may in fact have unmasked GA to be the actual AMD phenotype. Further work is of course required to investigate this intriguing hypothesis.

**Effects of genetic and non-genetic risk factors in a changing retinal landscape**

AMD is driven by rare as well as common genetic risk factors. Our work has contributed significantly to revealing its genetic architecture (Fritsche et al., 2013; Fritsche et al., 2015), including the roles of fibrillin (Ratnapriya et al., 2014), fibulin 5 (Stone et al., 2004), complement proteins and their regulators (Ennis et al., 2008; Gibson et al., 2012; Sofat et al., 2012), ApoE (McKay et al., 2011) and HLA (Goverdhan et al., 2005), as well as the roles of genes in chromosome 6p21.3 (Cipriani et al., 2012). Our findings have also provided molecular insights into how a variant cystatin C associated with AMD could impair the RPE (Paraoan et al., 2004; Ratnayaka et al., 2007b) and how extracellular proteins such as SPARC alter the RPE-BrM interface (Ratnayaka et al., 2007a). Thus far however, the molecular/biochemical consequences of most risk genes and how they might alter tissues in the outer retina remains to be determined. AMD is also driven by non-genetic or environmental risk factors such as nutrition and smoking (Chew et al., 2013; Chiu et al., 2014; Woodell and Rohrer, 2014), which play significant roles in predisposing the senescent retina to disease. Hence, as conditions in the retina gradually alter with age, different risk factors exert their influence singly and in concert to bring about irreversible change. How precisely these factors influence the development of retinopathy, and to what extent, are as yet unclear and may largely depend on the individuals’ genetic/non-genetic backgrounds. For instance, the deposition of cholesterol in the retina is associated with developing AMD (Rudolf and Curcio, 2009; Klein et al., 2010). Several cholesterol-linked genes such as CETP, ABCA1, LIPC and ApoE have also been correlated with AMD, but not necessarily the amounts of cholesterol deposited in retinas of individuals carrying mutations or isoforms of these genes. Hence, genetic influences on how much retinal cholesterol becomes deposited may be affected further by an individuals’ diet, the presence of other proteins as well as cholesterol clearance/turnover mechanisms. The formation/deposition of advanced glycation end products (AGEs) in the senescent retina also represents a further shift towards disease (Glenn and Stitt, 2009). Other factors such as Zn2+ and Fe3+ that accumulate in the RPE-BrM interface, for which there appears to be no obvious genetic link (Hahn et al., 2003; Lengyel et al., 2007), further contributes to alter the biophysical landscape of the retina. The accumulation of Amyloid beta (A) in the ageing retina represents another such risk factor, and the focus of this review.

A is a family of misfolding proteins correlated with neurodegenerative diseases such as AD. A is generated from sequential cleavage of the ubiquitously expressed amyloid precursor protein (APP) and is secreted extracellularly by neurons (Jarrett et al., 1993; Hardy and Selkoe, 2002). Given its central role in AD, histological data showing high quantities of A persistently accumulating in aged/AMD retinas were somewhat unexpected, particularly since a genetic correlation between A and AMD is yet to be established. It is also possible that any genetic correlation is either weak or non-existent, and that A accumulation is akin to the deposition of other substances such as AGEs, Zn2+ and Fe3+, lipofuscin or cholesterol in aged/AMD retinas. Why this highly neurotoxic group of proteins consistently aggregate in the aging retina, and to what extent they contribute to AMD, remains to be fully addressed. Our interest also stems from the fact that A is fundamentally different to many of the aforementioned substances or compounds accumulating in aged retinas, as A appear to have the capacity to trigger/drive multiple disease pathways. For example, A is associated with inducing neuronal senescence (He et al., 2013), disrupting cell membrane integrity (Williams et al., 2010), altering neuronal long-term potentiation (Townsend et al., 2006), inducing synaptic loss (Lacor et al., 2007; Shankar et al., 2007) as well as inducing memory defects in animal models (Duff et al., 1996; Citron et al., 1997) amongst others. Our earlier work also revealed a pathway through which A causes neuronal death (Soura et al., 2012). Additionally, A has the capacity to activate both the alternative and classical complement pathways (Bradt et al., 1998), elicit neuroinflammation (Minter et al., 2016) as well as induce vascular permeability and angiogenesis (Jefferies et al., 2013). We consider this to be a key feature by which A potentially differs from other substances that aggregate in the senescent retina.

**A synthesis and assembly- insights into A accumulation in the senescent retina**

Differences in the way APP is cleaved yields a variety of A peptides of which A1-40 and A1-42 species are the most common (Benilova et al., 2012). A levels as well as the relative ratios of A1-40 vs. A1-42 are altered in AD patients (Hardy and Selkoe, 2002; Kuperstein et al., 2010). Moreover, A peptides may be modified by further enzymatic activity resulting in a mixture of >20 different A peptides. The process of A aggregation is described by the nucleation-dependent polymerisation model in which monomeric A assembles into dimers, trimers and oligomers followed by a switch to a faster phase where larger structures including fibrillar A are formed (Jarrett et al., 1993; Ward et al., 2000; Lee et al., 2011). The formation of smaller soluble A forms as well as larger insoluble A aggregates may be observed in-vitro (Bitan et al., 2005; Benilova et al., 2012). Importantly, these A form can also be isolated from AD brains (Hardy and Selkoe, 2002; Shankar et al., 2008; Noguchi et al., 2009). Oligomeric A has been shown to have a closer relationship with AD progression (McLean et al., 1999; Mc Donald et al., 2010) compared to A plaques which do not necessarily correlate with disease severity (Perrin et al., 2009). The potency of oligomeric A is illustrated by experiments showing how its ability to penetrate biological membranes diminishes as oligomers assemble to a fibrillar state (Williams et al., 2010). A compelling case can therefore be made for oligomeric A as key driver of degeneration, further supported by a plethora of new findings claiming the identification of various toxic A oligomers. Amyloid plaques have been proposed to exist in a dynamic equilibrium with oligomeric A resulting in a local spillover of neurotoxic A species in adjacent tissues (Tseng et al., 1999; DeMattos et al., 2002). Oligomeric A may also mediate effects at some distances from established plaques (Benilova et al., 2012) with the latter perhaps acting as a reservoir for toxic A species. For these reasons, we routinely utilize in-vitro preparations of oligomeric A in our experiments. Of the diverse A species, A1-42 in particular has garnered considerable attention, as the addition of a hydrophobic isoleucine and alanine residue at its C-terminus is thought to facilitate rapid aggregation compared to A1-40 (Kim and Hecht, 2005). A1-42 is also thought to provide a nucleus for subsequent fibril formation (Jarrett et al., 1993) and is associated with a plethora of neurodegenerative events (Selkoe, 2008; Benilova et al., 2012). For these reasons we and many others utilize oligomeric A1-42 in our experiments, although this may exclude putative effects of an as yet unidentified ‘toxic A species’ or indeed a ‘toxic A oligomeric soup’ consisting of several A species, which might exist in AD brains (Benilova et al., 2012). We employ a method of in-vitro A preparation used by Broersen and colleagues (Broersen et al., 2011) in which we are able to clearly visualise the progressive assembly of smaller, soluble A forms (Soura et al., 2012). The resulting A not only conforms to the expected size of A oligomers, but their authenticity has also been confirmed by utilizing antibodies that specifically detect A (Taylor-Walker et al., 2016). Concerns have been raised about the use of non-physiological A concentrations in experiments that range from levels in excess of 1M A to as high as 10-40M A. Researchers are however driven to these extremes due to issues including (a) requirements for high A concentrations which may be necessary for monomeric A to assemble into toxic oligomers, (b) attempts to recapitulate disease conditions in a matter of several hours or days that would otherwise require several decades, as well as (c) the fact that in-vitro generated A appear to be less toxic compared to A oligomers generated from cultured cells or brain tissues (Selkoe, 2008; Benilova et al., 2012). In our view, acute dosage using non-physiological A concentrations does not necessarily replicate chronic A exposure to which cells/tissues of the ageing retina are subjected to over many years. Hence, our studies use physiological A concentrations in the picomolar to nanomolar levels, and only as high as 1M A, which may be considered to realistically recapitulate chronic A exposure in native tissues. In reality however, the extent of A exposure in different tissues may vary considerably, and may even differ between groups of cells in a given tissue as conditions such as temperature, pH, the presence of metals and other proteins/lipids influence the speed of aggregation, as well as the availability of toxic A oligomers in a given locality (Atwood et al., 2003; Benilova et al., 2012). Hence, the experimenter can only hope to influence A assembly under in-vitro conditions, as once introduced into a biological system, the likelihood of controlling A becomes largely academic. Toxic A oligomers could therefore rapidly form and/or persist in a given micro-environment, whilst their assembly may follow an alternative fate in another. In this respect, cell cultures may be more amenable to manipulating A, as this becomes nearly impossible under in-vivo conditions.

**What is the role of A in the senescent retina?**

Histological data from human donor eyes and mouse tissues reveal specific locations where A accumulates in the outer retina. The focus of A deposition appears to be in the RPE and BrM, although it also aggregates in photoreceptor outer segments (POS) and within choroidal vessels (Hoh et al., 2010; Ohno-Matsui, 2011; Ratnayaka et al., 2015). Histological analysis has also revealed A within drusen organised into structures between 0.25-20m in diameter termed amyloid vesicles (Anderson et al., 2004; Luibl et al., 2006; Isas et al., 2010). The interior of these vesicles were organised into concentric ring-like layers with different electron densities and permeated with A immunoreactivity (Anderson et al., 2004). Drusen were found to contain one or more such amyloid vesicles, which constituted a significant portion of their volume. The authors suggest that multiple amyloid cores could indicate that these drusen may have coalesced from several smaller drusen (Anderson et al., 2004), as is indeed the case since drusen is known to alter shape and change over time (Sarks et al., 1999). Furthermore, their findings showed A to be in retinas with moderate to high amounts of drusen, suggesting that A might also be associated with progressive stages of AMD (Anderson et al., 2004). The presence of A within drusen has also been confirmed histologically by several other groups (Mullins et al., 2000; Johnson et al., 2002; Dentchev et al., 2003; Luibl et al., 2006; Isas et al., 2010). Use of antibodies which recognised distinct A structures indicated oligomeric rather than monomeric or fibrillar A to form the core of drusen, in close proximity to the inner collagenous layer of BrM (Luibl et al., 2006; Isas et al., 2010) and the site of drusen biogenesis (Rudolf et al., 2008). Moreover, oligomeric A appeared to form the majority of A within drusen (Luibl et al., 2006; Isas et al., 2010). In contrast, fibrillar A was found to be predominantly localised to the periphery of amyloid vesicles (Johnson et al., 2002; Anderson et al., 2004; Luibl et al., 2006; Isas et al., 2010). Assembly of A as senile plaques in AD brains is considered to act as a scaffold onto which components such as ApoE, complement proteins, immunoglobulins, fibroblast growth factor, AGE products, as well as prion proteins amongst others subsequently bind (Armstrong, 2009). A also has the propensity to bind metals such as Zn2+ and Fe3+ (Atwood et al., 2003), whilst effects of post-translational modification and cross-linking has been shown to decrease its solubility and increase resistance to proteases (Smith et al., 1994). These events may significantly alter the biophysical properties of senile plaques over long periods. A similar process might also occur in the senescent retina, in which oligomeric A forms a scaffold in proximity to the inner collagenous layer of BrM, around which drusen constituents subsequently accumulate. This possibility is consistent with the hypothesis that chronic local inflammatory and immune-mediated events at the RPE-BrM interface play critical roles in drusen formation and hence in the development of AMD (Anderson et al., 2010). Ample evidence for complement proteins interacting with amyloid vesicles have also been reported by immunofluorescence studies in fixed donor AMD tissues (Johnson et al., 2002). Other types of interactions between drusen components and A have also become evident. For instance, the exposure of RPE cells to iron was shown to upregulate APP and increase A synthesis (Guo et al., 2014), whilst senescent C57BL/6 wildtype mice fed a high cholesterol diet developed sub-RPE A deposits (Wang et al., 2012b). There is also strong evidence to suggest that A effects are not limited to early AMD but may also manifest in late stages. For instance, exposure of cultured RPE to high A levels resulted in elevated secretion of pro-angiogenic VEGF, whilst human umbilical vein endothelial cells spontaneously formed tubes when exposed to conditioned media from A-treated RPE (Yoshida et al., 2005). Furthermore, injection of A into zebrafish eyes resulted in rapid proliferation of retinal capillaries (Cunvong et al., 2013). A treatment of cultured RPE also induced cellular senescence and created a pro-inflammatory microenvironment (Cao et al., 2013). Collectively, this evidence suggests that A could play an important role in progressive stages of AMD. Much like a spider at the centre of a complex web, A appears to have the capacity to trigger and/or drive multiple mechanistic pathways associated with early as well as late-stage AMD.

**The molecular basis of A in AMD**

Although fixed eye tissues from aged/AMD donors provide unequivocal histological insights into the importance of A in retinal degeneration, they are largely unsuitable for studies investigating more dynamic aspects of A-mediated retinal pathophysiology. Fortunately, we are able to call upon the aid of rodent models, as their pattern of retinal A aggregation is similar to that observed in donor human eyes. For instance, A has been shown to accumulate in the RPE-BrM interface (Hoh et al., 2010; Wang et al., 2012b), POS and in retinal and choroidal vessels of wildtype C57BL/6 mice (Hoh et al., 2010), as well as in the basal side of RPE and in the choroid of Long-Evans rats (Zhao et al., 2015). A also accumulates in sub-RPE deposits and choroidal neovascular lesions in ApoE4-HFC transgenic mice (Malek et al., 2005; Ding et al., 2008), in outer and inner plexiform retinal layers of APPswe/PS1E9 transgenic mice (Perez et al., 2009), as well as in the retinal ganglion cell (RGC) and inner nuclear layers of Tg2576 transgenic mice (Dutescu et al., 2009). These findings demonstrate that mice represent a powerful model with which A-mediated retinal pathology could be studied under realistic in-vivo conditions. To take advantage of this, we developed a murine model where a specific type (oligomeric), species (A1-42) and A quantity (nanomolar) was introduced into living retinas in a controlled manner. In the past, others have introduced A into the retinal environment by means of an intravitreal injection (Walsh et al., 2002; Walsh et al., 2005; Howlett et al., 2011). However, we reasoned that introduction of A via a subretinal route would recapitulate conditions in the senescent retina and associated tissues more accurately, particularly as the RPE is considered to be the major source of A synthesis/secretion in the posterior eye (Ohno-Matsui, 2011; Ratnayaka et al., 2015). The importance of RPE for A synthesis has been confirmed by immunohistochemistry which showed the expression of APP and the A-producing enzyme -secretase in these cells (Yoshida et al., 2005), and by cell culture and ELISA methods which showed that isolated mouse RPE secreted higher A levels compared to cells from younger animals (Wang et al., 2012a). As described earlier, histological evidence from donor AMD tissues as well as wildtype and transgenic mice also confirmed the importance of the RPE for retinal A production. Our studies showed the expression of the three alternatively-spliced mRNA APP isoforms; APP695, APP751 and APP770 (Ponte et al., 1988; Tanzi et al., 1988) in retinal and RPE homogenates (Figure 2A-B), revealing the breadth of APP expression in tissues of the outer retina (Lynn et al., 2016). For our mouse experiments, we used nM A quantities, in contrast to doses as high as 40M A used intravitreally in the past (Walsh et al., 2002; Anderson et al., 2009; Fisichella et al., 2016). We also used the highly toxic oligomeric A instead of fibrillar A used by others (Howlett et al., 2011) which is considered to be less pathogenic. We utilized wildtype C57BL/6 mice as their dark retinal pigmentation allowed better visualisation of incipient as well as advanced stages of retinal pathology. One week after subretinal A injection, mice displayed normal retinal functions compared to vehicle injected controls as measured by full-field electroretinogram (ERGs). This was reassuring since it indicated a functional living retina unimpaired by surgical procedures or gross retinal pathology. Subretinal A was introduced in a manner such that only half of the retina became exposed, whilst the majority of the retina remained unexposed and therefore acted as an internal control. Furthermore, the overall area of A exposure was relatively small, typically 20-30% of the total retina, which recapitulated the likeness of localised retinal pathology that develops in the macula of AMD patients (Lynn et al., 2016). We note that early changes in AMD patients are similarly undetectable by full-field ERGs (de Oliveira Dias et al., 2016). However, whilst full-field ERGs detected no changes, immunohistochemical analysis revealed a very different picture (Figure 2C-D). We observed pigment abnormalities via funduscopy which were correlated with hyperplastic RPE and disrupted RPE-BrM; indicative of a compromised BRB. Mice also showed loss of POS (Lynn et al., 2016; Ratnayaka and Lynn, 2016), which collectively constituted salient features of early-intermediate AMD (Davis et al., 2005; Khandhadia et al., 2012). We are therefore confident that our acute A-injection mouse model mimics subtle as well as specific features of AMD.

We next sought to determine specific locations in which A could potentially aggregate in retinas of these mice. One week after injection, A was observed in the RPE-BrM interface, on POS as well as in the outer plexiform layer, inner nuclear layer and RGC (Lynn et al., 2016; Ratnayaka and Lynn, 2016; Taylor-Walker et al., 2016). This pattern of A aggregation showed a striking correlation with areas of pathology indicated by parallel histological studies. Although pathology of the inner retina is not associated with AMD per se, areas of A aggregation in the retina corresponded to neurons/cell-layers known to be immunopositive for APP and/or A (Guo et al., 2007; Dutescu et al., 2009; Hoh et al., 2010; Kipfer-Kauer et al., 2010; Dasari et al., 2011; Koronyo-Hamaoui et al., 2011; Wang et al., 2011). We found it surprising that exogenously delivered human A1-42 should also colocalize to these specific retinal locations. We wanted to establish whether this implied that certain retinal layers or types of retinal neurons were in some way susceptible to A. To study this at single cell-resolution we turned to cultured retinal neurons, as such in-vitro approaches provide the necessary control and allows use of powerful microscopes to address this question. Chronic exposure to 1M A1-42 resulted in rapid internalisation of oligomeric A which remained sequestered within neurons for several days, whilst larger A forms appeared to aggregate outside neurons (Taylor-Walker et al., 2016) (Figure 2E-F). This pattern of rapid cellular internalisation by small, soluble A species such as oligomers was in keeping with the known biophysical characteristics of oligomeric A (Williams et al., 2010; Benilova et al., 2012). Similarly A assembly, in particular that of A1-42, into larger structures was also consistent with the reported behaviour of this aggregate-prone A species (Jarrett et al., 1993; Ward et al., 2000; Lee et al., 2011). It is possible that numerous, small retinal A deposits, which we detected in our mouse model were initially formed in this manner. On-going experiments in our laboratory are investigating the aetiology of such retinal A aggregates. Our work also revealed that internalised A transiently impaired the microtubule-associated protein 2 (MAP-2) (Taylor-Walker et al., 2016), which normally maintains the spacing/gapping between adjacent microtubules (Teng et al., 2001; Harada et al., 2002). MAP-2 is also important for dendritic arbours (Vaillant et al., 2002) and contributes to long-term dendritic stability (Sudo and Baas, 2010). Chronic A exposure could impair activities of neuroretinal cells during neuro-morphogenesis which occurs in post-mitotic neurons (Rich et al., 1997; Reye et al., 2002; Grandel et al., 2006; Pow and Sullivan, 2007). Indeed, MAP-2 is found co-localised to inner segments of abnormal photoreceptors with neurite sprouts, tortuous axons and abnormal nuclei in donor human AMD retinas (Pow and Sullivan, 2007). If chronic A exposure induces MAP-2 impairment in retinal neurons, it could have implications for other neuronal activities including intracellular transport of cargos and synaptic plasticity. For instance, our earlier work revealed how cytoskeletal elements such as actin can modulate neurotransmission which underpins plasticity (Ratnayaka et al., 2011). We suggest that such subtle A-driven mechanisms might be at play over many years to irreversibly compromise retinal neurotransmission. These may not always be evident, but if retinal neurons are targeted by A in this manner, it may explain why some AMD patients present primary photoreceptor pathology in the absence of any RPE damage (Bird et al., 2014).

**Future directions**

Here we have reviewed the latest literature related to A deposition in the senescent retina, and discussed potential mechanisms by which it could impair retinal health based on our findings. The acute A-injection mouse model we developed allows us to study dynamic aspects of A-mediated pathophysiology in living retinas which cannot be studied using difficult to source fixed donor tissues. This in-vivo model also allows us to control the dosage and types of A to which the retina is exposed, as well as to mimic the subtleties and specificities of the AMD phenotype to a remarkable extent. The results show striking similarities to early-intermediate AMD, reproducing features such as pigment abnormalities, disrupted, hyperplastic RPE, BrM breakage as well as loss of POS within a relatively short period. As such, our acute A-injection mouse model represents a powerful tool in the arsenal to study AMD. Our discoveries into how A impairs retinal neurons at single-cell resolution provide the first insights into how the retina may become damaged over decade-long A exposure. On-going experiments will delve further into functional consequences to reveal novel mechanisms which could form the basis for future treatments. Our experimental work and those of others thus reveal the extent to which A in donor aged/AMD retinas could actually contribute to disease (Figure 3). The potential implications of removing retinal A was demonstrated in an elegant study where ApoE4-HFC transgenic mice were rescued from visual defects by systemic A-immunotherapy (Ding et al., 2008; Ding et al., 2011). The importance of A in diagnosing AMD has also become evident, as plasma A levels were recently demonstrated to be a promising biomarker for those suffering from late-stage AMD (Guymer et al., 2015). Taken together, these developments could reveal an as yet poorly defined but important disease mechanism in the retina, which could also be used to predict AMD and provide insights into its rate of progression.

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