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Review

Re-emphasizing early Alzheimer's disease pathology starting in select entorhinal neurons, with a special focus on mitophagy

Asgeir Kobro-Flatmoen [a,b,1,**], Maria Jose Lagartos-Donate [c,1], Yahyah Aman [c], Paul Edison [d], Menno P. Witter [a,b,**], Evandro F. Fang [c,e,*]

[a] Kavli Institute for Systems Neuroscience, Centre for Neural Computation, and Egil and Pauline Braathen and Fred Kavli Centre for Cortical Microcircuits, MTFS, Olav Kyrres Gate 9, NTNU Norwegian University of Science and Technology, 7489, Trondheim, Norway
[b] KG.Jebsen Centre for Alzheimer's Disease, NTNU, 7489, Trondheim, Norway
[c] Department of Clinical Molecular Biology, University of Oslo and Akershus University Hospital, 1478, Lørenskog, Norway
[d] Neurology Imaging Unit, Department of Medicine, Imperial College London, London, UK
[e] The Norwegian Centre on Healthy Ageing (NO-Age), Norway

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ABSTRACT

The entorhinal-hippocampal system contains distinct networks subserving declarative memory. This system is selectively vulnerable to changes of ageing and pathological processes. The entorhinal cortex (EC) is a pivotal component of this memory system since it serves as the interface between the neocortex and the hippocampus. EC is heavily affected by the proteinopathies of Alzheimer's disease (AD). These appear in a stereotypical spatiotemporal manner and include increased levels of intracellular amyloid-beta Aβ (iAβ), parenchymal deposition of Aβ plaques, and neurofibrillary tangles (NFTs) containing abnormally processed Tau. Increased levels of iAβ and the formation of NFTs are seen very early on in a population of neurons belonging to EC layer II (EC LII), and recent evidence leads us to believe that this population is made up of highly energy-demanding reelin-positive (RE+) projection neurons. Mitochondria are fundamental to the energy supply, metabolism, and plasticity of neurons. Evidence from AD postmortem brain tissues supports the notion that mitochondrial dysfunction is one of the initial pathological events in AD, and this is likely to take place in the vulnerable RE + EC LII neurons. Here we review and discuss these notions, anchored to the anatomy of AD, and formulate a hypothesis attempting to explain the vulnerability of RE + EC LII neurons to the formation of NFTs. We attempt to link impaired mitochondrial clearance to iAβ and signaling involving both apolipoprotein 4 and reelin, and argue for their relevance to the formation of NFTs specifically in RE + EC LII neurons during the prodromal stages of AD. We believe future studies on these interactions holds promise to advance our understanding of AD etiology and provide new ideas for drug development.

1. Introduction

Alzheimer's disease (AD) is a progressive and fatal neurodegenerative disease of the central nervous system (CNS). It is the most common cause of dementia and currently affects over 45 million people worldwide (ADI, 2019). Clinically, AD is characterized by an insidious onset with progressive deterioration of cognitive abilities, typically beginning with declarative memory impairment which worsens as impairments in judgement and reasoning also become apparent (Canter et al., 2016; Kerr et al., 2017). The classical neuropathological hallmarks of AD are extracellular amyloid-beta (Aβ) plaques, mainly consisting of Aβ aggregates, and intracellular neurofibrillary tangles (NFT) containing the aberrantly hyper-phosphorylated microtubule associated protein Tau (p-Tau). These hallmarks are preceded or accompanied by several other pathologies, including but not limited to increased levels of intracellular $A\beta_{1-42}$ peptides ($iA\beta_{1-42}$), mitochondrial dysfunction, neuroinflammation, synaptic dysfunction and degeneration, as well as neuronal loss (reviews in (Canter et al., 2016; Kerr et al., 2017; McKhann

* Corresponding author at: Department of Clinical Molecular Biology, University of Oslo and Akershus University Hospital, 1478, Lørenskog, Norway.
** Corresponding authors at: Kavli Institute for Systems Neuroscience, Centre for Neural Computation, and Egil and Pauline Braathen and Fred Kavli Centre for Cortical Microcircuits, MTFS, Olav Kyrres Gate 9, NTNU Norwegian University of Science and Technology, 7489, Trondheim, Norway.
E-mail addresses: asgeir.kobro-flatmoen@ntnu.no (A. Kobro-Flatmoen), menno.witter@ntnu.no (M.P. Witter), e.f.fang@medisin.uio.no (E.F. Fang).
[1] Co-first authors.

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et al., 2011)). Despite half a century of extensive research on AD, there is still no treatment to slow or halt the disease. This unfortunate reality is at least in part due to an incomplete understanding of the molecular mechanisms at play in the structures initially affected by the disease, such as the entorhinal cortex (EC). Here, we aim to highlight the major advancements in our knowledge of AD development and progression with a focus on neurons in EC and mitochondrial dysfunction. We suggest that a resurgence in research on EC in the AD field is necessary to fully unveil the etiology of AD, and we anchor our suggestion on the facts that EC is essential for declarative memory and also constitutes one of, if not the first cortical region affected by AD pathology. We emphasize the need for further research on pathways connecting iAβ, NFTs, reelin, apolipoprotein 4 (ApoE4), and defective mitophagy, and suggest new approaches for the development of novel therapeutic strategies for AD.

2. Anatomy of the medial temporal lobe and the entorhinal cortex

Current knowledge supports the view that EC LII contains the first cortical neurons to develop pathological changes related to AD (Braak and Braak, 1991; Kaufman et al., 2018). In this section, we consider anatomical and molecular features of these EC LII-neurons and suggest reasons for their vulnerability. We begin by pointing out the location of EC and the associated structures that make up the medial temporal lobe (Fig. 1), before considering its pivotal contribution to declarative memory.

2.1. The medial temporal lobe

The medial temporal lobe (MTL) is a brain region with anatomically related structures that are essential for conscious memory of facts and events, termed declarative memory. The MTL, as recognized in primates, occupies the ventral and medial surface of the temporal lobe (Nieuwenhuys et al., 2008; Squire et al., 2004). Several structurally and functionally distinct areas considered essential components of the declarative memory system reside in MTL: from medial to lateral these include the hippocampus (the dentate gyrus and cornu ammonis (CA) 1–3), the subiculum, the pre- and parasubiculum, EC, the perirhinal cortex, and, posterior to the latter, the parahippocampal cortex (Franko et al., 2014), which in rodents is known as the postrhinal cortex (Cappaert et al., 2015). We provide detailed depictions of these anatomical structures and nomenclatures in Fig. 1(A–C). Knowledge about the functions of MTL memory structures relies heavily on knowledge gained from observational studies on cases with anatomically restricted lesions (Crick and Koch, 2003). Importantly, the study of anatomical circuit-level features has gained traction over the past two decades (van Strien et al., 2009), and is now at a point enabling detailed answers to questions concerning the function and malfunction of different MTL memory structures and their sub-domains, especially in EC (Nilssen et al., 2019).

EC provides the hippocampus with information derived from multiple parts of the cerebral cortex, collectively referred to as the association cortex, while also returning hippocampal-processed information back out to the association cortex (McKhann et al., 2011). The term “association cortex” signifies an essential difference from primary sensory cortex in that the latter is where sensory impressions first register at the cortical level, while association cortex receives information from primary cortex, or other parts of association cortex, and further process this into increasingly more complex representations (Yeo et al., 2011). The hippocampus sits atop the cortical hierarchy, and, largely by way of EC, information from all major regions of the neocortex converges in the hippocampus. This fits well with the notion that information processing in the hippocampus is crucial for several aspects of declarative memory (reviewed in (Eichenbaum and Cohen, 2014)) and, by virtue of providing the input, it comes as no surprise that the integrity of EC is also important for many of those aspects of declarative memory (Van Hoesen et al., 1991; Velayudhan et al., 2013b). Unlike a simple hub, however, EC does more than simply route information. Below we briefly consider some of the unique functional contributions of EC.

Within the past 20 years, EC went from being somewhat of a black box area mediating cortical-hippocampal connectivity, into a structure displaying remarkable functional properties. This is owed to a dedicated effort to understand its anatomy that subsequently helped realize experiments devoted to function. In brief, the finding that neurons in the dorsal hippocampus of rats show spatial modulation, most notably observed in so-called ‘place cells’, led scientists to search upstream into EC aiming to find neurons potentially providing the signals required for the observed spatial modulation in the hippocampus. Though this search initially met with little success, subsequent attempts informed by anatomical knowledge showed the existence of spatially modulated neurons in a dorsomedial part of EC. This part, generally referred to as the medial entorhinal cortex (MEC; see section 2.3 for further details) contains so called grid cells (Hafting et al., 2005) which, through their spatially modulated firing, tessellate any given 2D environment in hexagonal fields. It was subsequently revealed that MEC harbors several functional cell types that together enable navigation (reviewed in (Moser et al., 2017)). The MEC’s counterpart, generally referred to as the lateral entorhinal cortex (abbreviated LEC in rodents), was recently found to contain neurons capable of tracking relative time (Tsao et al., 2018), while other neuron types in LEC encode the present and past position of objects (Tsao et al., 2013), or code for specific objects (Suzuki et al., 1997). Efforts to uncover anatomical details continue to guide highly successful research into the functional properties of entorhinal neurons.

MTL and even more in particular EC degeneration occurs early in the pre-clinical stages of AD, and is seen most dramatically in LII-neurons (Kordower et al., 2001; Velayudhan et al., 2013a), typically followed by degeneration involving parts of the hippocampal formation (Scheff et al., 2006). Increasingly we have noticed studies on AD that do not take into account the anatomical specificity of the disease, which is likely to lead to missed opportunities for the field. We therefore believe it worthwhile to rehash some central aspects of the entorhinal-hippocampal anatomical layout and connectivity. First, however, it is important to realize that most of our understanding about this connectivity derives from work on species other than humans, the majority being on rodents (Cappaert et al., 2015; Witter, 2012), though much information has also been acquired through studies on cats (Van Groen et al., 1986; Witter and Groenewegen, 1984, 1986) and non-human primates (Insausti and Amaral, 2008; Witter et al., 1989). Of note, recent studies using high-resolution functional and structural magnetic resonance imaging (MRI) have so far substantiated the existence of the same pathways when studied in the human brain (Maass et al., 2015; Navarro Schroder et al., 2015; Zeineh et al., 2017).

2.2. Basic entorhinal architecture: histology

Six separate layers can be identified in human EC, with parallels the situation in other mammals (Fig. 1C). The outermost layer, adjoining the pia, is most often referred to as layer I. Here, comparably few neurons reside and those present are small. LII, also referred to as the pre-alpha layer, takes on a remarkable structural pattern by showing highly clustered neurons separated by relatively large gaps. These clusters, often called cell-islands, encompass many large neurons including star-shaped, fan-shaped and pyramidal-shaped types, plus smaller neurons of both bi- and multipolar types. The latter, smaller neurons also reside in the gaps between cell islands. Medium-large pyramidal neurons are the most prominent type in layer III, while bi- and multipolar neurons are also present. Unlike neocortical areas, EC lacks an internal granule layer, such that what is recognized as layer IV in EC is essentially a thin acellular strip, also called the lamina dissecans (“the cut lamina”), because it separates the deep from the superficial layers. Layer V

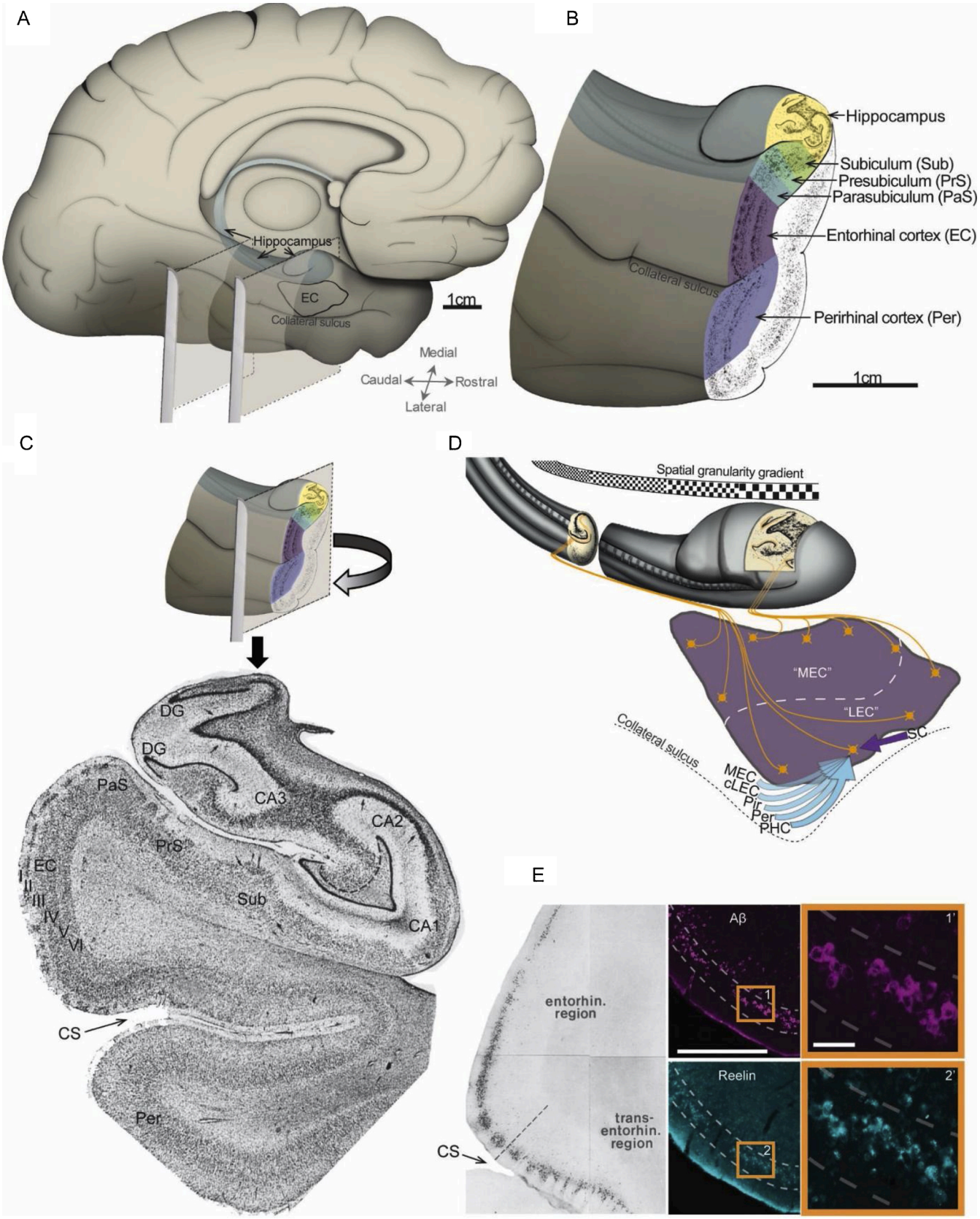

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Fig. 1. Structures of the medial temporal lobe (MTL) and focus on entorhinal cortex layer II (ECL-II) and the early pathology affecting this layer.
(A) Cartoon showing the position of the human left MTL along with the approximate extent of EC, observable after resection of the cerebellum and the brainstem. **(B)** MTL cut out of the left hemisphere as indicated in (A). The basic anatomical layout of the declarative memory structures of MTL are illustrated in the coronal plane at the level of the hippocampal uncus. **(C)** Nissl stained coronal section corresponding to the level indicated by the knife cut shows the cytoarchitecture of different MTL structures. The section was adapted from figure 24.8 in(Insausti and Amaral, 2012), with permission. Abbreviations: CA1, 2 and 3 = Cornu Ammonis 1, 2 and 3; CS = Collateral sulcus; DG = Dentate Gyrus; remaining abbreviations are explained in (B). **(D)** Illustration showing the hippocampus and EC L-II isolated from surrounding structures and the fact that neurons close to the collateral sulcus (CS, black dashed line) target the caudal hippocampus, while neurons located increasingly further away from CS target increasingly more rostral parts of HF, such that those furthest away from CS have projections confined to the rostralmost/uncal part of HF. The increased spatial granularity in more caudal parts of HF is illustrated by a pixelation scale. Note that though for EC only L-II neurons are indicated, their pattern with respect to rostral vs caudal connectivity with the hippocampus holds true for all layers of EC. Also note that in the anterolateral EC ("LEC", corresponding to rodent lateral entorhinal cortex), a single principle neuron apparently integrates inputs from several different cortical areas, alongside modulatory inputs from subcortical structures. Abbreviations: MEC = medial entorhinal cortex; cLEC = contralateral LEC; Pir = piriform cortex; Per = perirhinal cortex; PHC = Parahippocampal cortex; SC = subcortical inputs **(E)** (left panel) Micrograph from Braak and Braak (1991)(Braak and Braak, 1991) showing how early NFT formation (stage 3) in EC LII is most pronounced in the domain located close to CS, which is also the domain where the first NFTs arise (Braak and Braak, 1991). (mid- and right panels) Colocalization (double-immuno) of $iA\beta_{1-42}$ (pink) and reelin positive (RE+) neurons (blue) in corresponding domain of EC LII of a rat model of AD (scalebars: 500 μm for micrographs, 50 μm for insets), adapted from the Ph.D thesis of Asgeir Kobro-Flatmoen, see also (Kobro-Flatmoen et al., 2016)).

contains pyramidal neurons of medium to large size in its superficial half, and smaller pyramidal neurons in its deep half. In layer VI, which adjoins the white matter, there is a mix of neuron types, including pyramidal and multipolar neurons, and the overall appearance is more heterogeneous (Braak, 1980; Insausti et al., 1995).

Importantly, the organization of EC is not uniformly laminar. Rather, the domains located close to the collateral sulcus (Fig. 1A) are well-developed, but with increased distance from the collateral sulcus the lamination gradually appears less developed. Thus, at the point furthest from the collateral sulcus, the individual layers are poorly organized and difficult to discriminate (Insausti, 1993). This organizational feature was recently shown to largely overlap with that of the chemo-architecture (Kobro-Flatmoen and Witter, 2019), which is further discussed in the following section.

2.3. Basic entorhinal architecture: topography

As previously mentioned, EC consists of two main subdivisions, the lateral and medial entorhinal cortices (LEC and MEC, respectively), corresponding to the older division of Brodman's Area 28a and 28b, respectively. There are strong arguments for this two-part division. First, a long line of research in many species shows that the two parts can be easily differentiated cytoarchitectonically. Second, in rodents, their respective projections to the hippocampal dentate gyrus terminate in different zones (Cappaert et al., 2015; Witter, 2007). In primates, our current understanding of these features is less clear cut, since multiple subdivisions are more readily observed based on cytoarchitectonics, and the projections to the dentate gyrus predominantly show a more diffuse terminal distribution (Insausti and Amaral, 2012; Witter et al., 1989). More recently, connectivity patterns have been proposed to identify the two subdivisions, and these seem to be consistent for rodents and primates (Maass et al., 2015; Navarro Schroder et al., 2015; Witter and Amaral, 2021).

The fundamental architecture of EC is highly similar across species. The above described gradient in laminar differentiation tracks with a chemo-architectonic gradient, with multiple neurochemical markers increasing or decreasing in expression level depending on the distance from the collateral (primates) or rhinal (rodents) sulcus (Kobro--Flatmoen and Witter, 2019). An example of this is the protein reelin. This protein, known to serve as an extracellular matrix protein, mainly locates to small interneurons scattered throughout the cerebral cortex. However, for reasons that remain poorly understood, in EC reelin is present in a large population of LII principal neurons (Figs. 1E and 2 A). Moreover, the amount of reelin shows a marked gradient: high-levels are present in LII neurons close to the collateral sulcus, whereas neurons located increasingly further away from the collateral sulcus contain gradually less, until the portion located furthest away contains little to no reelin (Kobro-Flatmoen and Witter, 2019). While the functional significance of this feature remains unknown, findings suggest that high-level reelin neurons might be more prone to accumulate Aβ (Kobro-Flatmoen et al., 2016), a point we will return to in section 3.

2.4. Relevance of entorhinal connectivity: focus on LII neurons

The past four decades has seen a strong line of evidence supporting the notion that EC LII-neurons are the first cortical neurons to degenerate in the course of AD (Braak and Braak, 1991; Gomez-Isla et al., 1996; Hyman et al., 1984; Kordower et al., 2001; Olsen et al., 2017), a point we return to in the subsequent section. Meanwhile, studies on EC anatomy have reached an impressive, yet still incomplete level of circuit detail (Cappaert et al., 2015). Here we refrain from considering all of the complex EC circuitry, and instead focus on that of LII. Though we recognize that a key role might stem from the concerted activity of multiple elements within the EC circuitry, the current text is aimed at providing context for the connections of LII neurons and their position in the cortical hierarchy, aligned to our current understanding of the early stages of AD.

Multimodal sensory and highly processed olfactory inputs converge in EC (reviewed in (Cappaert et al., 2015)). Among these inputs, it has been known for quite some time that two major ones originate in the parahippocampal (postrhinal in rodents) and perirhinal cortices, structures strongly involved in enabling aspects of declarative memory. These structures are thought to mediate information concerning, respectively, the external environment (often conceptualized as a "where-pathway"), alongside changes occurring in that environment, including those resulting from navigating it (often conceptualized as a "what-pathway"; (Ritchey et al., 2015). Recent work on LEC shows that a given LII principal neuron receives convergent inputs from both the parahippocampal/postrhinal cortex and the perirhinal cortex, as well as from a number of other cortical domains (Doan et al., 2019). In fact, *a single LEC principle neuron* integrates input from at least five different, highly active cortical areas (Burwell and Amaral, 1998; Doan et al., 2019; Insausti et al., 1987; Jones and Witter, 2007; Kondo and Witter, 2014; Mathiasen et al., 2015; Vaudano et al., 1991), alongside modulatory inputs from subcortical structures (Fig. 1D). A similar situation likely holds true for principal cells in LII of MEC (Witter et al., 2017).

Upon reaching the LII neurons and the network in which they are embedded, information undergoes further processing thought to yield high-order representations of the environment and its changing nature. At this level, EC LII-neurons are positioned to communicate extensively with neurons throughout layers I–V (Cappaert et al., 2015). Within LII, the most prevalent type of projection neuron is the reelin-expressing stellate (MEC; (Canto and Witter, 2012b)) or fan cell (LEC; (Canto and Witter, 2012a)), so named for the shape of their large dendritic trees. These neurons send their axons to the hippocampus, a fact we will return to shortly, but they also give rise to an extensive axonal plexus within LII. Despite this, these neurons do not directly communicate with each other; rather, they connect only indirectly, via inhibitory interneurons

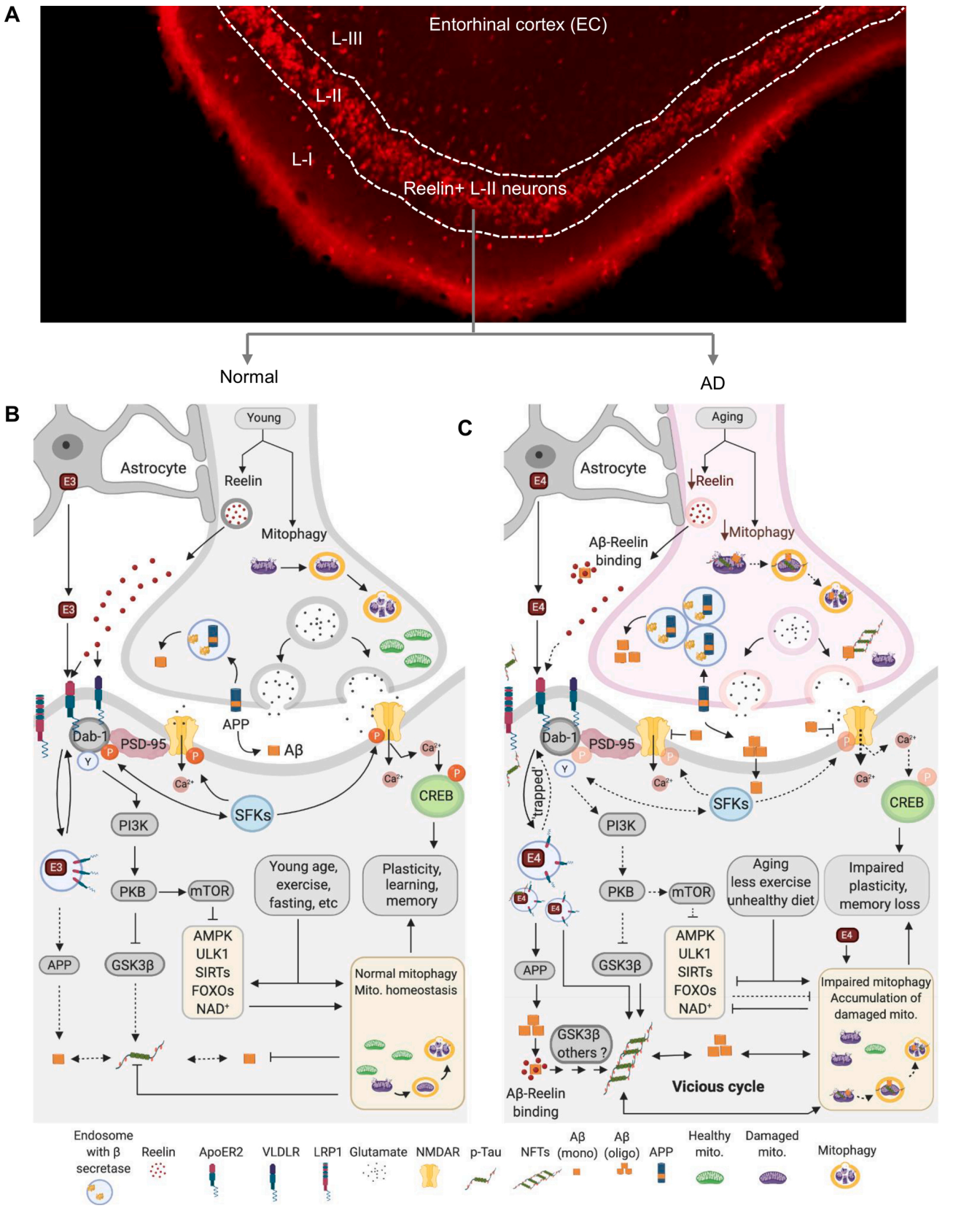

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Fig. 2. Proposed mechanism of the occurrence of the earliest AD pathology in entorhinal cortex (EC) L-II. **(A)** Immunofluorescently labeled reelin-positive neuronal population in L-II of rat EC. **(B-C)** Schematic representation of the signal transduction cascade generated by reelin under normal physiological conditions (B) vs AD (C). **(B)** Under normal conditions, including in young individuals, reelin is highly expressed. Reelin binds to lipoprotein receptors, such as ApoER2 and VLDLR, inducing activation of Disabled 1 (Dab1), an adapter protein. Activated Dab1 induces activation of Src family kinases (SFKs) that potentiate tyrosine phosphorylation of Dab1, which in turn activates Phosphoinositide 3-kinase (PI3K) and subsequently protein kinase B (PKB). PKB activation inhibits the activity of GSK3β, thereby reducing p-Tau and promoting microtubule stability. PKB also activates mTOR-dependent processes which promote the outgrowth of dendrites and balances mitochondrial biogenesis and autophagy. Changes of mTOR activity affect the activities of ULK1, AMPK, sirtuins (SIRTs), FOXOs, and NAD^+. In addition, ApoER2-reelin complex is coupled to NMDAR signaling through PSD95. Reelin-activated SFK phosphorylates NMDAR and potentiates NMDAR-Ca^{2+} influx. Influx of Ca^{2+} activates the transcriptional regulator, CREB, through which expression of genes important for synaptic plasticity and neurite growth are potentiated. CREB-regulated genes encode proteins important for learning and memory. Astrocyte- and neuron-derived ApoEs bind to ApoER2 and are constitutively internalized. Upon reelin signaling, ApoER2 also undergoes endocytosis. In the cases of ApoE2 and ApoE3, ApoER2 is efficiently recycled to the cell membrane; this is impaired in the case of ApoE4. In healthy young individuals, mitophagy effectively clears damaged mitochondria, ensuring a healthy mitochondrial pool in the high-energy demanding axon terminals; this enables normal neuronal function and neuronal plasticity. Mitophagy also eliminates intracellular $iA\beta_{1\text{-}42}$ and pathological Tau proteins. **(C)** Ageing is the primary driver of AD with multiple molecular mechanisms involved, including age-dependent reduction of reelin and impaired mitophagy (also autophagy). In prodromal AD, reduced reelin in the EC L-II neurons impairs the control of the ApoER2/VLDLR-Dab-1-PI3K-PKB-GSK3β axis, leading to pTau. Furthermore, $iA\beta_{1\text{-}42}$ is increased in the EC L-II neurons, possibly due to increased production (via ApoE4-dependent trapping, detailed below), and reduced clearance by impaired mitophagy/autophagy. $iA\beta_{1\text{-}42}$ may bind reelin and thereby reduce levels of signaling competent reelin, in turn impairing PKB mediated inhibition of GSK3β and thus increasing p-Tau. ApoE4 may accentuate this as it tends to get trapped in endosomes along with its lipoprotein receptors, likely mainly ApoER2. This in turn further reduces reelin-signaling, boosting the cascade leading to p-Tau. In concert, ApoE4, trapped in endosomes, increases transcription of APP and thus production of $iA\beta_{1\text{-}42}$, and thereby completes a vicious cycle, whose end product for the affected neurons are NFTs. Furthermore, reduced PKB-activity also inhibits mTOR activity, impacting on mitochondrial homoeostasis and autophagy. Moreover, ApoE4 sequesters ApoER2 in intracellular compartments and reduces the NMDAR phosphorylation in response to reelin in the postsynaptic neuron, leading to impaired neural plasticity. In line with the age onset of AD, age-dependent mitophagy impairment causes accumulation of damaged mitochondria, which further exacerbates AD pathology, including shortage of energy supply, inflammation, oligomerization of $iA\beta_{1\text{-}42}$ and pathological Tau proteins, finally leading to impaired LTP and neuronal plasticity, and neuronal loss. Individuals carrying *ApoE4* may have exacerbated mitophagy impairment since ApoE4 inhibits TFEB-dependent regulation of autophagy- and lysosome-related genes. Dashed lines and faint phosphorylation symbols indicate blunted signaling capacity. Abbreviations: Aβ, amyloid-β; AD, Alzheimer's disease; AMPK, 5' AMP-activated protein kinase; ApoE, apolipoprotein E; ApoER2, ApoE receptor 2; Ca^{2+}, calcium ion; CREB, cAMP response element-binding protein; Dab1, disabled 1; EC, entorhinal cortex; FOXOs, Forkhead box O (FOXO) transcription factors; GSK3β, glycogen synthase kinase 3β; LTP, Long-term potentiation; NMDAR, N-methyl-D-aspartate receptor; PKB, protein kinase B; SIRTs, the NAD^+-dependent deacetylates sirtuins; SFKs, SRC family tyrosine kinases; ULK1, unc-51 like autophagy activating kinase 1; VLDLR, very-low-density lipoprotein receptor; low-density lipoprotein receptor-related protein 1 (LRP1). Dashed arrows indicate impaired induction/activation.

(Couey et al., 2013; Nilssen et al., 2018). Thus, reelin-expressing stellate and fan neurons in LII are likely subject to strong inhibitory control by their peers.

The reelin-expressing neurons in EC LII originate the main projection to the dentate gyrus and fields CA3/CA2 (Kitamura et al., 2014; Varga et al., 2010). A second organizational feature of these projections relevant to the present discussion is that they have a graded topographical organization. Work on monkeys, corresponding to that in rodents, shows that projections originating from neurons close to the collateral sulcus terminate in the posterior portion of the hippocampus. Projections originating increasingly further away from the collateral sulcus target portions increasingly anterior in the hippocampus (Witter and Amaral, 1991; Witter et al., 1989). Functional MRI studies in humans indicate that this graded topographical organization relates to the observation that the most posterior hippocampal portion functionally deals with fine-grained spatial representations, while anterior parts deal with coarse grained spatial representations (Evensmoen et al., 2015, 2013). We illustrate this EC LII-hippocampal connectional feature, along with the spatial granularity observed in the hippocampus in Fig. 1D.

3. AD proteinopathies and the early involvement of EC

Continued failures in anti-AD drug development demonstrate the need for a better understanding of the etiology of AD, including potentially specific molecular changes occurring in anatomically distinct regions (Canter et al., 2016; Hou et al., 2019; Kerr et al., 2017). Histochemistry-based neuron number estimations indicate very early neuron loss in EC LII (Arendt et al., 2015; Giannakopoulos et al., 1996; Gomez-Isla et al., 1996; Hof et al., 2003; Hyman et al., 1984; Kordower et al., 2001), and this is reflected in loss of EC LII terminals in the hippocampus (Scheff et al., 2006), and is corroborated by reports on *in vivo* volume reduction of EC (Dickerson et al., 2001; Holbrook et al., 2020; Juottonen et al., 1998; Killiany et al., 2002; Kulason et al., 2019; Olsen et al., 2017; Tward et al., 2017; Velayudhan et al., 2013b). Congruently, knock-in of mutated human APP in mice, resulting in Aβ-pathology, disrupts MEC grid cell remapping and thus shows how an impaired circuit mechanism may lead to early navigation impairment in AD (Jun et al., 2020). In the subsequent sections we attempt to account for possible pathological molecular mechanism underlying early changes in the disease process of AD, maintaining a focus EC LII neurons.

3.1. AD proteinopathies and their consequences

A major hypothesis regarding the cause of neurodegeneration in AD is the misfolding and aggregation of toxic proteins (proteinopathies). In AD, the best-known misfolded proteins give rise to extracellular Aβ-plaques, and intracellular NFTs (Ballatore et al., 2007; Canter et al., 2016; Fang et al., 2019b; Goedert, 2015; Kerr et al., 2017; Selkoe and Hardy, 2016). Aβ-plaques are principally made up of a mixture of abnormally fibrillated and poorly soluble Aβ-peptides, and NFTs stem from aberrantly hyperphosphorylated microtubule associated protein (MAP) Tau (p-Tau). Though the physiological function of Aβ peptides is still poorly understood, their origin is well known. They originate through sequential cleavage of a larger precursor protein known as the amyloid precursor protein (APP). Depending on the enzymatic complexes, the subcellular milieu and even the substrate itself, the cleavage can yield a mixture of Aβ-peptides ranging from 37 to 43 amino acids (reviewed in (Steiner et al., 2018)). Of these, the $A\beta_{1-42}$ variant is not only the first to be deposited into plaques but also the most toxic (Iwatsubo et al., 1995, 1994).

In the case of humans, alternative mRNA splicing of the *microtubule associated protein tau*-gene (*MAPT*-gene) results in six common Tau isoforms that range from 352 to 441 amino acids. These six isoforms differ with respect to whether they have three vs. four instances of a repeat sequence in the C-terminal half, and whether or not they have one or both of two possible inserts close to the N-terminus. The repeat sequences along with flanking portions make up the microtubule-binding domains. Notably, these are also the domains forming the core of Tau filaments (Goedert et al., 1989) and (reviewed in (Goedert, 2015)). For Tau, unlike what is the case for Aβ peptides, several physiological functions have been demonstrated. Tau binds to and serve as a protective layer shielding the surface of microtubules from severing enzymes. Furthermore, the Tau-microtubule interactions regulate the capacity of other MAPs as well as of molecular motors to interact with microtubules

(Monroy et al., 2018; Siahaan et al., 2019; Tan et al., 2019). Since these are crucial functions to the integrity of neurons, they help explain why malfunctioning Tau, including p-Tau, can lead to impaired subcellular cargo mobility, microtubule breakdown, neuronal stress and neurodegeneration. A long held notion is that Tau stabilize microtubules (Mandelkow and Mandelkow, 2012), however, recent results contradict this and rather indicate that Tau functions by enabling a domain on each microtubule to remain labile (Qiang et al., 2018).

It is important to realize that while Aβ plaques and NFTs form the disease-defining pathologies as seen upon post mortem examination, these are late-stage pathologies in the course of AD (Dubois et al., 2014; Duyckaerts et al., 2009), although increasingly sophisticated positron emission tomography (PET) analyses now enable detection of amyloid aggregates in living subjects before clinical symptoms are present. Experimental and clinical evidence suggests that an imbalance between the production and clearance of Aβ peptides, with that of the $A\beta_{1-42}$ form being the most critical, is an early, and possibly disease-initiating factor (Muller et al., 2019). A concern often raised against the importance of Aβ, is the fact that *Aβ plaques* show only a low-to-moderate correlation with cognitive decline (reviewed in (Nelson et al., 2012)). It has been reported that several individuals with substantial Aβ-plaque burdens in their brains appear cognitively unimpaired as measured by standardized clinical tests (Price et al., 2009). However, non-fibrillated assemblies of Aβ that are soluble in non-denaturing solutions (soluble Aβ), which by definition are not in the form of plaques, show a strong correlation with cognitive decline (Koss et al., 2016; Lue et al., 1999; McLean et al., 1999; Naslund et al., 2000; Steinerman et al., 2008). This may be taken to indicate that the visually conspicuous Aβ-plaques are only a part of the Aβ pathology and that substantial toxic assemblies of Aβ-peptides exist outside of plaques (Benilova et al., 2012).

The classical disease-defining pathological lesions of AD arise in a predictable spatiotemporal pattern across the brain. Highly sensitive immunohistochemical analyses show that Aβ-plaques initially arise in neocortical areas, including the temporal cortex, in the form of occasional small focal groups of diffuse plaques. These initial plaques thus define *a neocortical stage*. Next, Aβ-plaques appear in allocortical and periallocortical structures of MTL. In this stage, hippocampal field CA1 (allocortex) is consistently affected, while EC (periallocortex) is affected in a large majority of cases. Additionally, there is often involvement of other parts of the hippocampal region. Aside from MTL, plaques are often observed in the cingulate gyrus, the insular cortex and the amygdala. Due to the consistent involvement of CA1, this second stage constitutes *the allocortical stage*. Following these initial two stages, plaques arise in subcortical nuclei and the brainstem (stages 3 and 4) and eventually even the cerebellum (stage 5). During the latter stages of disease development, a continuous increase in plaque-load occurs in allo-, periallo- and neocortical regions (Thal et al., 2002, 2000). Recent work based on uptake of the radiopharmaceutical compound florbetapir, as measured using PET-imaging, suggests that the neocortical areas most consistently involved during the initial parenchymal aggregation of Aβ include the posterior cingulate and orbitofrontal cortices, along with or immediately followed by the precuneus (Palmqvist et al., 2017).

Regarding Tau, current evidence shows that it's pathological forms arise in neurons in LII of the domain of EC located close to the collateral sulcus, sometimes referred to as the transentorhinal region (Kaufman et al., 2018); this is also the initial cortical area to develop full-blown NFTs (Braak and Braak, 1991) Fig. 1E). Pre-tangle pathology may present in the locus coeruleus before that of EC (Braak et al., 2011), but recent work suggests that the capacity for pathological Tau-seeding likely begins in EC rather than in the locus coeruleus (Kaufman et al., 2018). Tau pathology next appears downstream to EC in the distal part of hippocampal field CA1 and in a portion of the subiculum that may constitute the proximal subiculum (see figure 9 stage II in (Braak and Braak, 1991)). Eventually, much of the neocortex becomes affected. A recent study based on a large cohort of older adults provides strong support for the notion that these histochemical findings reflect an actual spatiotemporal spread of pathological Tau. Functional magnetic resonance imaging (fMRI) was used to measure functional connectivity, and this was contrasted with PET-imaging measuring uptake of the radiopharmaceutical compound 18F-flortaucipir, binding to pathological Tau. The authors found that regions with a stronger functional connectivity to the Tau-vulnerable domain of EC located close to the collateral sulcus were also the regions that contained most pathological Tau (Adams et al., 2019). This study thus seems to offer a glimpse into events occurring in the human brain at a stage leading up to AD. However, how might this seeding of pathology occur?

In vitro studies point to p-Tau oligomers being taken up by cells via clathrin-mediated endocytosis, micropinocytosis and direct membrane fusion, enabling transfer of p-Tau from cell to cell (Calafate et al., 2016; Evans et al., 2018; Katsinelos et al., 2018). More recent evidence indicates that Tau can also spread transsynaptically without being misfolded, although it has been suggested that spreading might be enhanced by misfolded p-Tau (Hallinan et al., 2019; Sanders et al., 2014). Neurons in EC are permissible to misfolding of Tau, a non-universal characteristic since it was shown to be absent in the striatum (Wegmann et al., 2019). A large body of work thus supports the hypothesis that p-Tau and NFTs arises in LII in the portion of EC located close to the collateral sulcus and subsequently spreads along anatomical connections, gradually bringing about neurodegeneration and failure of the affected networks. Where then, does Aβ come in?

Mutations in the *MAPT* gene that result in changes in the level of p-Tau do not lead to AD but can lead to frontotemporal dementia (reviewed in (Goedert et al., 2012)). The fact that several *APP* mutations cause AD, all by increasing total Aβ or $A\beta_{1-42}/A\beta_{1-40}$, while one known *APP* mutation that lowers production of Aβ protects against AD, provides strong evidence that Aβ is central to the disease. However, the onset of AD is likely not only about Aβ. Bolstered by novel techniques and laboratory models, the joint importance of Aβ and p-Tau in causing neuronal damage has been extensively investigated in recent years, with intriguing results. For example, Aβ oligomer-induced synaptotoxicty is dependent on CAMKK2-AMPK-mediated Tau phosphorylation (Mairet-Coello et al., 2013), while p-Tau reduction inhibits Aβ-mediated defects in axonal transport of mitochondria (Vossel et al., 2010). Thus, it appears that p-Tau and Aβ are both important for setting off AD, and the molecular mechanisms governing their interactions need to be determined.

3.2. $iA\beta_{1-42}$ accumulation in EC LII-neurons may affect reelin-signaling to impact Tau

In a transgenic rat model for AD, expressing human APP with two well-known mutations, we recently showed that in EC one of the two major neuronal populations of LII, namely the RE + population, is the first EC population to accumulate $iA\beta_{1-42}$. Moreover, *preliminary data* from human AD-subjects indicate that this might hold true for the actual disease as well (Kobro-Flatmoen et al., 2016). Intriguingly, both the amount of reelin and the amount of $iA\beta_{1-42}$ are highest in the portion of EC located towards the collateral sulcus, thus mirroring the pattern of the initial formation of NFTs (Fig. 1E). At least in the case of reelin this feature is present across mammals, including humans (reviewed in (Kobro-Flatmoen and Witter, 2019)).

Soluble assemblies of Aβ show a strong correlation with cognitive decline (Koss et al., 2016; Lue et al., 1999; McLean et al., 1999; Naslund et al., 2000; Steinerman et al., 2008). Although the main toxic species among these assemblies remains elusive, current evidence suggests that assemblies in the oligomeric-protofibrillar range are central to the disease (Benilova et al., 2012), most notably those made from $iA\beta_{1-42}$ owing to their neural toxicity, even in the nanomolar range (Cizas et al., 2010; Cleary et al., 2005; Walsh et al., 2002). Importantly, in humans, rodent models and primary neurons, APP cleavage yielding Aβ-peptides is known to occur inside neurons in endosomal multivesicular bodies (Takahashi et al., 2004, 2002), though minor amounts are likely present

in the Golgi apparatus and in mitochondria (reviewed in (Gouras et al., 2010; Kerr et al., 2017)). Also, studies of human subjects show that neurons accumulate iAβ_{1-42} before plaque pathology is present (Cataldo et al., 2004; Gouras et al., 2000; Ohyagi et al., 2005; Pensalfini et al., 2014) while the oligomerization of Aβ likely begins intracellularly (Oddo et al., 2006; Takahashi et al., 2004; Walsh et al., 2000). How might those vulnerable EC LII-neurons fit with this evidence?

The presence of reelin in EC LII projection neurons is an atypical feature in that reelin mainly associate with scattered interneurons throughout the cortex (Kobro-Flatmoen and Witter, 2019), and reelin has been proposed to be associated with high levels of synaptic plasticity (Beffert et al., 2005). It is further known that reelin signaling via the apolipoprotein E receptor 2 (ApoER2) and the very low-density lipoprotein-receptor (VLDLR) leads to inhibition of glycogen synthase kinase 3β (GSK3β), the main Tau kinase (Beffert et al., 2002; Hiesberger et al., 1999) (Fig. 2B). The effect of reelin signaling upon Tau is also evident *in vivo*, as reelin-deficient mice exhibit increased levels of p-Tau (Hiesberger et al., 1999; Ohkubo et al., 2003). A related line of work *in vitro*, shows that in the presence of Aβ_{1-42}, reelin fails to assume its signaling competent form, which leads to increased Tau phosphorylation (Cuchillo-Ibanez et al., 2013). Moreover, in samples taken from human AD brains, reelin co-immunoprecipitates with Aβ (Cuchillo-Ibanez et al., 2016). In line with this, we have reported evidence for an intracellular co-localization of reelin and iAβ_{1-42} in RE + EC LII neurons (Kobro-Flatmoen et al., 2016). In addition to reelin-binding to ApoER2 and VLDLR, another *LDLR* gene family member, namely low-density lipoprotein receptor-related protein 1 (LRP1), is a recently discovered regulator of endocytosis implicated in the spread of Tau (Rauch et al., 2020). Thus, these *LDLR* gene family members provide for an intriguing association between Aβ_{1-42}, reelin and Tau. Based on these findings we hypothesize that a pathological build-up of iAβ_{1-42} in EC LII-neurons impairs the reelin signaling-cascade and that this, likely via reduced binding of reelin to ApoER2, can lead to selective hyperphosphorylation of Tau in these neurons (Fig. 2C). We further suggest that this is likely a slowly progressing event, in part kept in check by compensatory mechanisms until some threshold of pathological, seeding-competent Tau is reached. This eventually sets into motion a cascading induction of pathological Tau into downstream connected areas.

An obvious challenge to this hypothesis comes from the fact that even though our work on the AD rat model provides evidence that early accumulation of iAβ_{1-42} in EC begins in RE + LII-neurons, these *model* neurons do not develop NFTs nor do they show any obvious signs of neurodegeneration. This is vastly different from that seen in human EC LII in AD-subjects. One possible reason for this discrepancy might be that the development of pathological Tau under physiological conditions, taking place within the confines of a cell with a working lysosomal-endosomal system, requires an incubation time of several years or even more than a decade. That is far beyond what is offered by a rodent model where animals can typically be analyzed at ages up to two years. Interestingly, a recent study on rhesus monkeys reported that compared with young adults, aged individuals (27–38 years old) with memory impairments have a reduction of RE + LII neurons in the anteriolateral region (the LEC homologue), which is not the case for aged monkeys with an intact memory (Long et al., 2020). This is in line with our notion that a reduction of functional reelin may be among the earliest pathological events in AD (Fig. 2C, upper part).

3.3. The Apolipoprotein E4 risk-factor is linked to reelin signaling

The reports on reelin signaling being mediated by ApoER2- and VLDLR receptors, mentioned in the previous section, is of interest since Apolipoprotein E (ApoE), which is known to carry cholesterol and other lipids to their appropriate targets in the brain, is strongly linked to AD (Xian et al., 2018). ApoE exists in slightly different isoforms as determined by the *ApoE* gene, which comes in any combination of three types on its two alleles, namely *ε2, ε3* or *ε4*. The majority of people have *ε3* biallelic, and this is therefore considered the baseline. The strongest genetic risk factor known for AD is carrying the *ε4* allele of the *APOE* gene (Schiepers et al., 2012). This risk factor is dose-dependent with subjects carrying *ε4* on one allele having a 2−3-fold increased risk, whereas those with *ε4* on both alleles have an increased risk amounting to 7−10-fold. On the other hand, carrying the *ε2* version is associated with a decreased risk for AD (Rasmussen et al., 2018).

Protein translated from an *ε4*-containing gene (*ApoE4*) results in several dose-dependent functional alterations with respect to interactions with and effects on other proteins. For example, while any version of ApoE will promote oligomerization of Aβ, ApoE4 dramatically increases this tendency, even more so when it is biallelic. The cause of this may be a direct interaction between ApoE and Aβ monomers which facilitates linking of the latter, in which ApoE4 somehow serves as the best scaffold (Hashimoto et al., 2012). Recent work further revealed how the different ApoE isoforms bind their receptor(s) in the same dose-dependent manner and trigger a signaling cascade driving transcription of APP to yield Aβ; note that it remains to be determined which of the ApoE receptors is responsible for this effect (Huang et al., 2017).

ApoE4 is also known to impair recycling of ApoE receptors along with NMDA receptors (NMDAR) and AMPA receptors (AMPAR) due to vesicular trapping in the endocytic transport machinery (Chen et al., 2010). As mentioned in the previous paragraph, reelin signals via binding either of two ApoE receptors, namely ApoER2 and VLDLR. Upon such binding, a signaling cascade is initiated resulting in increased Ca^{2+} influx through NMDAR plus insertion of additional AMPAR, thereby regulating synaptic strength (Qiu et al., 2006b), most likely by enabling synaptic potentiation (Chen et al., 2005), with molecular mechanisms possibly including Ca^{2+}-induced activation of cAMP response element-binding protein (CREB) and others (Bito et al., 1996; Kornhauser et al., 2002) (Fig. 2B). In fact, bath application of reelin onto hippocampal slices results in rapid induction of long-term potentiation (LTP) (Qiu et al., 2006b; Weeber et al., 2002), providing a strong indication that reelin serves a role in learning and memory (Qiu et al., 2006a). Importantly, the ApoE4-induced impairment of ApoER2 and VLDLR recycling causes reduced reelin-signaling and impaired LTP (Xian et al., 2018). From these findings, it is tempting to speculate that ApoE4 gets trapped in endosomes along with its receptors which can result in changes of multiple cellular pathways. If so, this could impact production of Aβ since not only does ApoE4 trigger increased transcription of APP to yield Aβ (Huang et al., 2017), but also, iAβ is known to derive from processing of APP in endosomes owing to endosomal expression of Aβ-generating γ-secretase, which generates Aβ_{1-42} and other Aβ peptides (Sannerud et al., 2016; Takahashi et al., 2004, 2002). Furthermore, trapped ApoE4 could reduce NMDAR phosphorylation in response to reelin in the postsynaptic neuron, leading to impaired neural plasticity (Fig. 2C). Collectively, current data suggest that a vicious loop is generated in RE + EC LII neurons whereby trapped ApoE4 increases production of iAβ_{1-42} while also impairing reelin signaling, which in turn drives up the signaling cascade leading to p-Tau and NFTs (Fig. 2). Note that it is likely the case that ApoE4 can affect Tau pathologies and neurodegeneration independently of Aβ pathology (Shi et al., 2017), but possible pathways involving reelin in this context is beyond the scope of this review.

3.4. Insights into neuron-specific vulnerability by transcriptomics

Recently, the single-nucleus RNA-sequencing (snRNA-seq) approach has enabled large scale transcriptomics of individual cells from post-mortem tissue, furthering our ability to identify factors underlying cell-specific vulnerability or resilience. Here we point to four such factors recently identified and associated with Tau pathology.

RAR-related Orphan Receptor B (RORB) is a marker recently found in a subset of excitatory neurons in the EC L-II taken from caudal domains (Leng et al., 2021). These authors found that Tau neuronal inclusions at Braak stages 1–2 were preferentially accumulated in

RORB-positive excitatory neurons in EC. A correlation was revealed between RORB reduction and changed transcriptional patterns encoding proteins associated with synapses and axons, the relevance of which awaits further study. Meanwhile, no major changes were found in any of the subpopulations of inhibitory neurons, regardless of layer (Leng et al., 2021). Whether the RORB-positive neurons partly or completely overlap with the RE + neurons in lateral domains of EC remains to be determined. Similarly, another protein linked to both the regulation of Tau accumulation and vulnerability of excitatory neurons is BCL2-associated athanogene 3 (BAG3), a facilitator of autophagy. BAG3 reduction exacerbates pathological Tau accumulation, while over-expression of BAG3 attenuates it (Fu et al., 2019). Another recently detected marker is polypyrimidine tract binding protein 1 (PTB), a splice factor which modulates the 3R/4R-Tau balance. PTB is dysregulated in

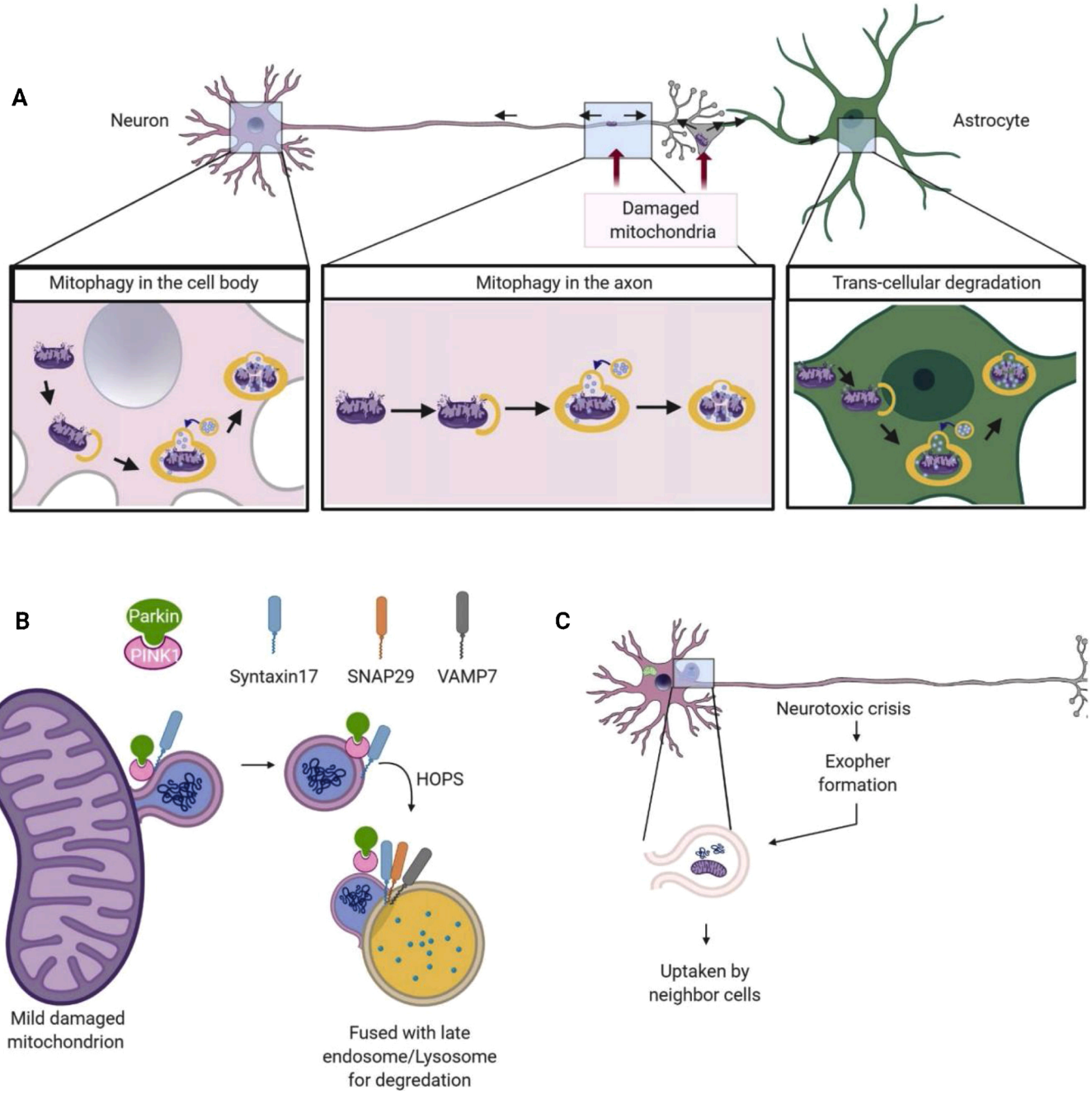


Fig. 3. A summary of mitochondrial clearance pathways in neurons. **(A)** Elimination of damaged mitochondria through classical mitophagy pathway at three different locations. Such locations include 1) the soma, where damaged mitochondria, either originally localized in the soma or moved here through retrograde transport from the distal axon, to be degraded via mitophagy; 2) in-site mitophagic degradation in the axon since lysosomes can be anterogradely transferred from the soma to the distal regions of the axon; and 3) 'trans-cellular mitophagy' in exogenous stress conditions in which neurons transfer damaged mitochondria to the adjoining astrocytes for mitophagic degradation. **(B)** The mitochondria-derived vesicle (MDV) pathway. Mildly damaged mitochondrion jettisons damaged cargo (e.g., oxidized mitochondrial proteins) to the lysosome for degradation. Syntaxin-17 is recruited to MVDs during budding with the participation of the mitophagy proteins PINK1 and Parkin. At the late endosome/lysosome, syntaxin-17 forms a ternary membrane-anchored SNARE complex with SNAP29 and VAMP7 to facilitate fusion in a manner dependent on the homotypic fusion and vacuole protein sorting (HOPS) tethering complex. MDV is finally degraded by the acidic lysosome. **(C)** Neurotoxic crisis induces budding of damaged subcellular components via large (4 μm diameter) membrane-bound vesicles (termed 'exophers'). The exophers are digested by the surrounding cells. Note that while this process has been reported in *C. elegans* neurons it is currently unknown whether it also happens in mammals.

AD, and it is suggested to contribute to sub-type specific neuronal vulnerability (Roussarie et al., 2020). A factor possibly conferring resilience is Methyltransferase Like 7B (METTL7B), which was found to be enriched in neurons known to be less vulnerable early in the disease process. In brain, METTL7B is virtually exclusive to excitatory neurons, and associates with ER and lipid droplets (Franjic et al., 2020). At a molecular level, METTL7B interacts with APP and seems to reduce the amount of Aβ-peptides, while no effects on Tau pathology was shown (Franjic et al., 2020).

Future work will hopefully bring us closer to a complete transcriptomic map of the neurons that degenerate first in AD, no doubt including the RE + EC LII neurons but likely also including select populations in the hippocampal formation. This should provide us with a better vantage point from which to refine our hypotheses about the onset of the disease.

4. Initial AD-related mitophagy impairment implicated in EC circuitry

Mitochondria are multifaceted subcellular organelles functioning as cellular powerhouses generating ATP that is essentail for the activities and survivual of neurons and glial cells. Mitochondria are also important for metabolic homeostasis, Ca^{2+} regulation, cellular signalling, neuroplasticity, and the arbitration of cell survival and death (reviewed in (Lou et al., 2019; Mattson et al., 2008)). If dysfunctional, mitochondria will impair neuronal activity (Han et al., 2020a) and can under certain conditions trigger neuronal death (Somayajulu et al., 2005). Neurons are a uniquely polarized and comparmented cell type, with a cell body, branched protoplasmic dentrites, and an axon, which together places a great demand on the efficient transport of mitochondria to different sub-cellluar regions for ATP produciton (Cheng et al., 2012). In particular, neurotransmission involves axonal presynaptic elements and dendritic postsynaptic elements which demand a high metabolic rate. This is only possible given healthy mitochondria (Sun et al., 2013). Under normal conditions damaged mitochondria are continuously identified and removed by mitochondrial quality control pathways (Fang et al., 2019b; Lin et al., 2017; Wang et al., 2011). Such pathways help ensure that healthy, energy-providing mitochondria prevail in critical domains.

One of the main processes for mitochondrial quality control is a specific type of autophagy, termed mitophagy. As the name implies, mitophagy involves recognition and ordered degradation of damaged or superfluous mitochondria (Lazarou et al., 2015). Different mitophagic pathways exist, suggesting redundancy, and while it is outside the scope of the current review to deal with each, we direct the interested reader to the following sources (Lou et al., 2019; Wang et al., 2019). In neurons, mitophagy is most prevalent in the soma, though it also occurs along the axon, including in the metabolically demanding axon terminals (Fig. 3A). Mitophagy plays a fundamental role in mitochondrial homeostasis, which is required to maintain efficient synaptic activity, regulate presynaptic strength – including the support of axon remodelling, and thus also to preserve the integrity of neuronal networks (Cheng et al., 2012; Heo et al., 2019; Sun et al., 2013; Zhou et al., 2016). It is therefore likely that even a partial loss of mitophagic capacity in structures subserving memory functions will cause functional deficits.

To what extent is mitophagy tied to AD? The possibility that mitochondrial function is pivotal in neurodegenerative diseases is a notion going back three decades (Linnane et al., 1989), and brain oxidative damage indicating mitochondrial impairment is indeed detectable already in individuals with mild cognitive impairment, of which the majority will go on to develop AD (Gan et al., 2014; Valla et al., 2006). Though recent results obtained in mice and postmortem human brain tissues specifically link age-dependent mitophagic/autophagic impairement to the development of AD (Bordi et al., 2016; Fang et al., 2019b; Lee et al., 2010), and also show that mitochondrial damage strongly correlate with tau pathology and granulovacuolar degeneration (Hou et al., 2020), even more pertinent results came from a recent study on a large cohort of AD-subjects (148 cases) which includes EC. This latter study tested whether mitochondrial impairment is broadly present early on, or whether such impairments aligns with the known early vulnerability of EC. Intriguingly, the data revealed mitochondrial impairment in EC already from the earliest pathological stage associated with AD, namely Braak stage 1 (Armand-Ugon et al., 2017). Subsequent to impaired mitochondrial activity in EC, this likely occurs in the hippocampus (Calkins et al., 2011; Fang et al., 2019b; Manczak et al., 2011), while neocortical areas become involved later in the disease process (Jadiya et al., 2019; Wang et al., 2005). Notably, mirroring the classical AD-pathology, the cerebellum is last to show signs of mitochondrial damage (Thubron et al., 2019).

Ageing, by far the single strongest risk factor of AD, is in itself associated with accumulation of impaired mitochondria. This likely occurs across animal phyla, including roundworms (Morsci et al., 2016; Palikaras et al., 2015), rodents (Zhao et al., 2020) and humans (Petersen et al., 2003). At least part of what is missing to separate normal ageing from AD in this context is a better understanding of the molecular mechanisms that maintain mitochondrial homeostasis in neurons.

4.1. Current understanding of mitophagic pathways

Here we give a brief overview of pathways that eliminate neuronal damaged mitochondria, including mitophagy, formation of mitochondria-derived vesicles (MDVs), and 'exophers'. For the interested reader, a more complete overview can be found in (Kerr et al., 2017; Lou et al., 2019).

Different components of the machinery involved with mitophagy have been proposed to cause its defect in AD, including impaired initiation of the mitophagic apparatus (Cummins et al., 2018; Fang et al., 2019b), reduced lysosome function (Lee et al., 2010; Martini-Stoica et al., 2018), and impaired transport of damaged mitochondria from presynaptic axonal elements to the soma (Han et al., 2020a). Transmitophagy is a more recently discovered mitophagic pathway, first reported in retinal ganglion cells (Davis et al., 2014). This pathway, in which the whole mitochondrion is removed, involves formation of mitochondria-laden axonal protrusions that undergo neuron-to-astrocyte transmission (Fig. 3A, right panel). Such cell-to-cell communication and resource exchange may contribute specifically to neuroprotection and also recovery following stressed conditions (Hayakawa et al., 2016). Another pathway is stimuli-defined and works by extruding small internal mitochondrial content to form MDVs tagged for lysosomal degradation (Fig. 3B; (Neuspiel et al., 2008). MDVs contain either a single membrane or double membrane based on different types of mitochondrial damage, and require both PINK1 and Parkin (McLelland et al., 2014). In the context of Parkinson's disease (PD), MDVs are known to trigger mitochondrial antigen presentation, linking mitochondrial dysfunction and autoimmune mechanisms (Matheoud et al., 2016), and, intriguingly, components involved in this pathway was recently linked to formation of early tau pathology and granulovacuolar bodies in the hippocampus (Hou et al., 2020). A third, recently discovered, waste-elimination system involves the formation of so-called exophers, large-diameter (up to 4 μm) membrane-bound vesicles that engulf mitochondria alongside various protein aggregates before being expelled by neurons and digested by the surrounding cells (Fig. 3C; Melentijevic et al., 2017).

In view of the complexity and high energy demand of the mammalian brain, and the resulting necessity for timely and efficient elimination of damaged mitochondria, it is not surprising that neurons operate with multiple mitochondrial maintenance pathways.

4.2. Impaired mitophagy and its possible relationship with Aβ, tau, and reelin

Impaired mitophagy induces accumulation of damaged mitochondria which can exacerbate and may in part trigger Aβ and Tau

pathologies (Fig. 4). The Fang lab and colleagues recently proposed and substantiated the hypothesis that defective mitophagy is a driver of AD-related pathology and disease progression (Fang et al., 2019a, b; Kerr et al., 2017). This proposition in based on observations of damaged mitochondria accumulated in postmortem hippocampi of AD patients, plus corresponding findings in AD subject-derived cortical-like neurons differentiated from induced pluripotent stem cells (iPSCs; (Fang et al., 2019b). Moreover, these findings are replicable in mouse models of AD, where mutations affecting both amyloid and tau processing impair mitophagy via inhibiting p-TBK1 and p-ULK1, kinases necessary for the initiation of the mitophagic machinery. Mutant tau may also inhibit the PINK1-Parkin-dependent mitophagy pathway via inhibiting the association of Parkin onto mitochondria (Cummins et al., 2018). Importantly, recent evidence demonstrates that reduced or defective mitophagy can in itself aggravate Aβ and Tau pathologies, as nicotinamide adenine dinucleotide (NAD^+)-dependent pharmacological restoration of mitophagy ameliorates pathology and prevents memory impairment in both roundworms and mouse models of AD (Fang et al., 2019b; Sorrentino et al., 2017). The mechanism following increased NAD^+ governing elimination of Aβ and p-Tau remains poorly understood, although recent findings allow for some tentative ideas. For example, NAD^{+-}enhanced mitophagy increases the microglia-dependent elimination of extracellular Aβ plaques (Fang et al., 2019b; Lautrup et al., 2019), while inhibiting NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome activation (Fang et al., 2019b). The latter is likely relevant since activation of the NLRP3 inflammasome increases levels of both Aβ and p-Tau (Heneka et al., 2013; Ising et al., 2019) summarized in Fig. 2C).

To our knowledge, no studies have addressed the potential effect(s) of reelin upon mitochondrial function. Likely relevant is that the reelin-ApoER2/VLDLR signaling pathway plays a necessary role in the growth and stability of the cytoskeleton evidenced by its activation of mTOR (leading to growth), its effect on actin cytoskeleton rearrangement (Chai et al., 2009), and its inhibition of p-Tau (Hiesberger et al., 1999) Fig. 2B). Altered microtubules will affect the motility patterns (e.g., speed, pause, and direction) of mitochondria and the supply of ATP, and this will likely impact on damaged mitochondria headed for mitophagic elimination (reviewed in (Sheng and Cai, 2012)). An example of altered mitochondrial motility patters is found in how migration of cellular Tau from the soma to the distal region of an axon is reversed in degenerating neurons (Dixit et al., 2008). In this latter case, somatic misallocation of Tau is associated with faulty kinesin-driven anterograde transport, while dynein becomes trapped in the soma (Fig. 4A, B). This results in energy deprivation due to mitochondrial insufficiency in the presynaptic axon terminal (Dixit et al., 2008). Work on roundworms substantiates this, showing how mutated Tau (e.g., A152 T) impair both anterograde and retrograde axonal transport of synaptic vesicles (Butler et al., 2019).

Aβ oligomers also impair mitochondrial motility via effects on GSK3β, casein kinase, actin polymerization, and by impairing NMDAR signaling (Decker et al., 2010; Zamponi et al., 2017). Interestingly, Tau reduction by homozygous- or complete KO (*Tau*$^{+/-}$ or *Tau*$^{-/-}$) prevents exogenous iAβ_{1-42}-induced defects in anterograde axonal movement of mitochondria in mouse primary hippocampal neurons (Vossel et al., 2010). A recent single-cell atlas on EC from AD-patients underscores the potential importance of this. Specifically, the authors found alterations of the transcription factor EB (TFEB), which is a master regulator of autophagy, lysosomal function, and phagocytosis, along with alterations in cellular pathways involved in autophagy and metabolism (Grubman et al., 2019).

5. Conclusions and outstanding questions

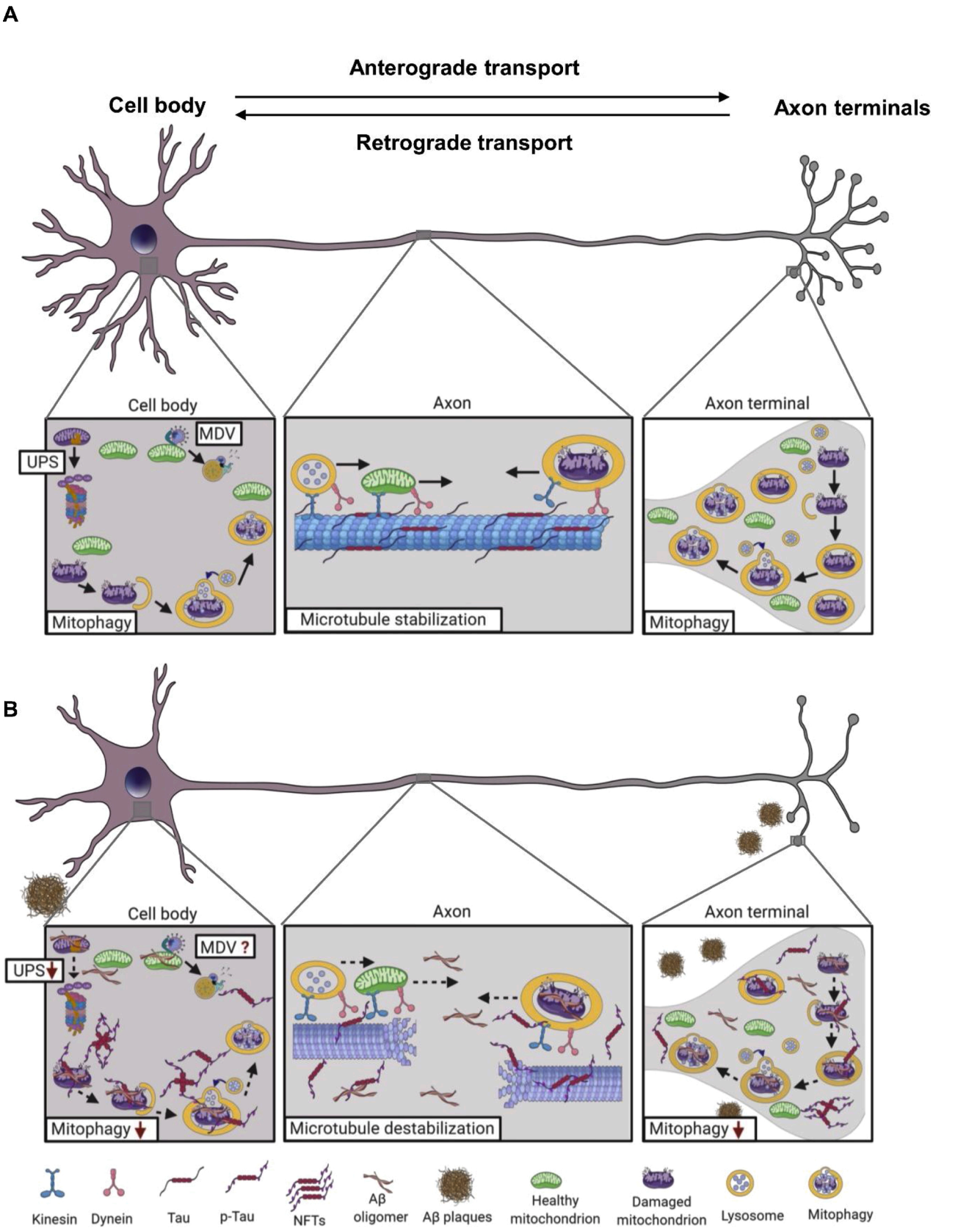
The substantial body of work involving EC along with recent progress from studies on AD patients and animal models enable us to propose a working model aiming to explain the molecular mechanisms of the earliest pathological events in EC LII (Fig. 5). Our model proposes that RE + EC LII-neurons have a very high metabolic rate supported by a highly active autophagic machinery, in which mitophagy plays a key role. Impairments in this machinery may drive neurons into a diseased state recognized as AD in the case of humans. We suspect a major *contributing* factor making RE + EC LII-neurons vulnerable to 'setting off' AD, is the high metabolic demand placed on them, stemming from their enormous dendritic trees (Canto and Witter, 2012a), which, for each cell, has to integrate information from at least 5 different cortical domains alongside subcortical inputs. This necessitates a particularly high degree of mitophagic activity and consequently, we suspect, renders a low tolerance for even partial impairment.

In concert with this our model posits that as mitophagy becomes less efficient with age, oligomeric iAβ_{1-42} slowly accumulates. As this happens, reelin interacts with iAβ_{1-42} to form reelin-iAβ_{1-42} complexes that impair efficient reelin-signaling, which in turn drives up the signaling cascade leading to p-Tau. This process is accentuated when expressing ApoE4 which tends to get trapped in endosomes along with its lipoprotein receptors. Importantly, as these lipoprotein receptors are also reelin-receptors, their trapping further impairs reelin signaling. This effect constitutes a second blow to the RE + LII-neurons, as the added impairment of reelin-signaling further promotes the cascade, which, via GSK3β, leads to p-Tau. The presence of ApoE4 accentuates this by its tendency to get trapped in endosomes, which drives up production of iAβ_{1-42}, and thereby completes the vicious cycle, finally resulting in NFTs in affected neurons (Fig. 5 **upper panel**). The damage incurred by mitochondria as the abovementioned pathological processes develop, possibly leads to mitochondrial hubs of inflammation. Notably, damaged mitochondria can activate the NLRP3 inflammasome, adding to the stress of the neuron. The fact that pathological Tau can spread in a prion-like manner, for example via LRP1, provides a clear explanation as to how the downstream hippocampus tends to become affected by NFTs following that of EC LII (Fig. 5 **upper panel**). Although our model is admittedly an incomplete one, it provides a testable explanation for why anteriolateral EC LII-neurons are frequently the first cortical neurons to degenerate in AD.

We propose testable hypothetic curves of iAβ and p-Tau/NFTs in EC L-II, accompanying disease severity. Our hypothesis posits that pathological accumulation of iAβ happens ahead of Tau pathology in EC LII; we also postulate that iAβ_{1-42} levels increase in EC LII ahead of Aβ_{1-42} accumulation in the CSF, suggesting a probable target of early biomarker development for AD. We further include the dramatic loss of EC LII neurons, starting during pre-clinical AD, (Fig. 5, lower panel).

Recent advancements in technology, including omics, genetic editing, and advanced labeling techniques have enabled the emergence of a new era with the potential to fully unveil the molecular events that initiate AD. This brings us closer to our common goal of developing an effective treatment for AD. However, this potential may only come to fruition if the focus of research is directed to where the disease is initiated, bearing in mind that AD is not a homogeneous brain disease. Here we have reviewed and argued for several strong lines of evidence pointing to EC LII as the likely home to the first *cortical neurons* to develop pathological changes associated with AD.

Among the outstanding questions in our model is that of the molecular mechanisms leading to damaged mitochondria in RE + EC LII-neurons, for example whether this mainly involves the canonical or either of the non-canonical mitochondrial elimination pathways. Considering the evidence for early mitochondrial damage in EC, further studies targeting mitochondrial homeostasis in the RE + LII-neurons may provide strategies for early diagnosis, and lead to concepts about early-stage interventions for AD. In view of the importance of the reelin-expressing stellate and fan neurons in LII, it will be important to determine how Tau, iAβ_{1-42}, and ageing affect the functional properties of these neurons, along with their status concerning mitochondrial quality and mitophagy. As important are future studies on the potentially unique molecular mechanisms and pathways underlying Tau, iAβ_{1-42}, ApoE4, and mitophagy interactions in the puzzling population



(caption on next page)

Fig. 4. Impaired mitochondrial clearance in AD neurons. **(A)** In a healthy neuron, mitochondrial homeostasis is maintained through different pathways, such as mitophagy, UPS^{mt}, and MDVs. Detailed molecular mechanisms of these pathways are visualized in Fig. 2. Neuronal mitophagy likely happens predominantly in the soma, with existence of in-site mitophagic degradation in the axon, including in the axon terminals. Mitochondria are transported from the soma to distal parts of neurons or to the sites of high energy demand along microtubule tracks using the motor protein complex kinesin (anterograde direction) or dynein (retrograde direction). In healthy neurons, damaged mitochondria in the distal regions of the axon can either be retrogradely transferred to the soma for mitophagic degradation or can be on-site degraded via mitophagy by lysosomes anterogradely transferred from the soma. **(B)** Impaired mitophagy and enhanced pathologies in the AD neurons. In AD neurons, increased accumulation of iA$\beta_{1\text{-}42}$ and p-Tau leads to abnormal trafficking in both directions. Aβ toxic oligomers and p-Tau are likely involved in microtubule destabilization, impairing retrograde transportation of damaged mitochondria from distal regions back to the soma for degradation, and may block anterograde transport of kinesin-tagged lysosomes to axons for on-site mitophagic degradation, resulting in impaired local degradation capacity. Impaired mitophagy is likely to exacerbate AD pathology by reducing the neurons' capacity for degradation of toxic proteins, including iA$\beta_{1\text{-}42}$ and p-Tau. While evidence exists for impaired mitophagy and compromised UPS^{mt} in AD, the status of MDV in AD is not known.

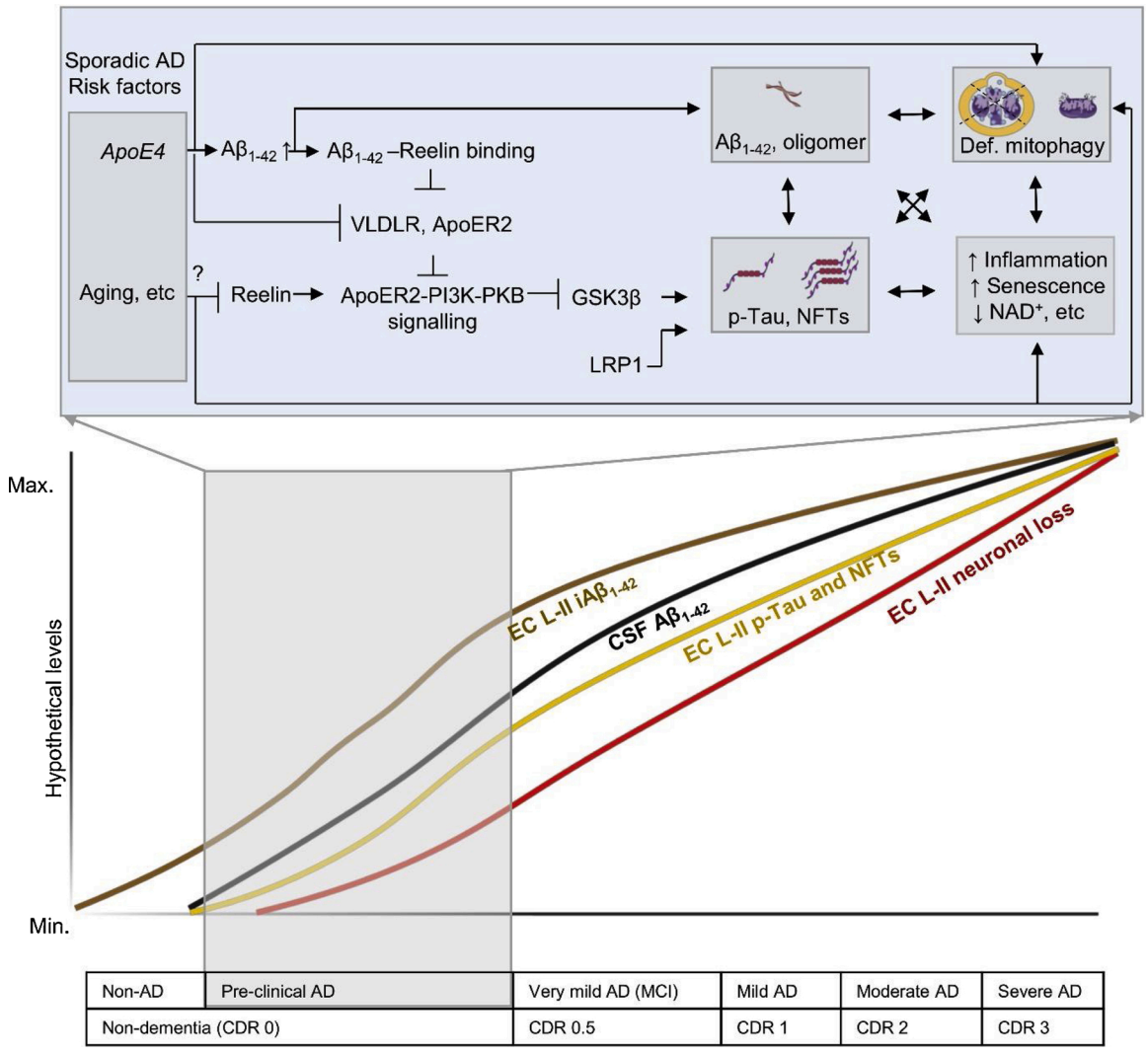


Fig. 5. Proposed molecular mechanisms of the earliest NFTs exist in the EC L-II region in relation to the time-course of cognitive, clinical, and hypothetically pathological changes.
We propose a time-course for changes of iAβ_{1-42}, tau pathology (p-Tau, NFTs), and neuronal loss in the EC L-II neurons, in which increased iAβ_{1-42} is the earliest event. Note that in this model, we propose that increased iAβ_{1-42} in EC L-II neurons occur before changes in CSF Aβ_{1-42}. **Upper panel:** Genetic AD risk factors, like *ApoE4* genotype, faciltate the generation of Aβ_{1-42}, which binds to reelin and impairs reelin-dependent inhibition of p-Tau and, ultimately, NFTs. ApoE4 also impairs autophagy/mitophagy via disrupting the autophagic machinary and lysosome functions. In addition to genetic risk factors, aging is the univeral and primary risk factor of AD, and includes reduced expression of reelin, a process whose molecular mechanisms remain to be determined. Furthermore, other age-dependent celluar changes, such as inflammation, senescence, NAD^+ reduction, and defective mitophagy/autophagy, contribute to AD initiation and progression. A vicious cycle, among Aβ pathology, Tau pathology, defective mitophagy, and other factors, exacerbates AD progression. **Lower panel**: it shows correlation between the clinical stages of AD (very mild, mild, moderate, severe) and clinical dementia rating (CDR) scores (0.5, 1, 2, 3).

of RE + EC LII-neurons.

For these purposes, the application of new, high-throughput techniques, including the use of single-nucleus RNA sequencing (Grubman et al., 2019) and chromatin immunoprecipitation combined with highly parallel sequencing (Marzi et al., 2018), will have to provide cell-type-specific and genome-wide information about EC from AD individuals. This approach promises to address pivotal questions in the field. It is thus encouraging to note that the field now has the technical

ability (Han et al., 2020b) to create a human EC-based comprehensive single-cell atlas, which may provide the resource required to achieve a solid understanding of the mechanisms that initiate AD.

Declaration of Competing Interest

The authors report no declarations of interest.

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