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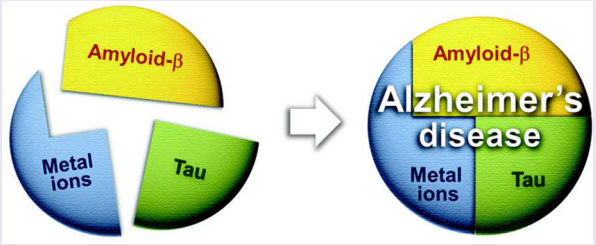
Untangling Amyloid-β, Tau, and Metals in Alzheimer's Disease

Masha G. Savelieff,[†] Sanghyun Lee,[†,§] Yuzhong Liu,[†,‡] and Mi Hee Lim*[,†,‡]

[†]Life Sciences Institute and [‡]Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109, United States

ABSTRACT: Protein misfolding and metal ion dyshomeostasis are believed to underlie numerous neurodegenerative diseases, including Alzheimer's disease (AD). The pathological hallmark of AD is accumulation of misfolded amyloid-β (Aβ) peptides and hyperphosphorylated tau (ptau) proteins in the brain. Since AD etiology remains unclear, several hypotheses have emerged to elucidate its pathological pathways. The amyloid cascade hypothesis, a leading hypothesis for AD development, advocates Aβ as the principal culprit. Additionally, evidence suggests that tau may contribute to AD pathology. Aβ and tau have also been shown to impact each other's pathology either directly or indirectly. Furthermore, metal ion dyshomeostasis is associated with these misfolded proteins. Metal interactions with Aβ and tau/ptau also influence their aggregation properties and neurotoxicity. Herein, we present current understanding on the roles of Aβ, tau, and metal ions, placing equal emphasis on each of these proposed features, as well as their inter-relationships in AD pathogenesis.



Alzheimer's disease (AD) is the most common neurodegenerative disease, accounting for 60−80% of all dementias.[1] Currently, it affects approximately 5.4 million Americans and 24 million people worldwide, and these numbers are expected to increase dramatically.[1,2] AD cases are categorized as early onset AD (EOAD) or late onset AD (LOAD), with 65 years of age as the cutoff.[1,3,4] EOAD, which only constitutes a fraction of all cases, has a strong genetic component.[1,3,4] Most cases are LOAD that is sporadic, although some genetic markers exist which increase the predisposition to develop AD.[1,3,4] AD-afflicted brains exhibit traits such as an overall decrease in size, a reduction in glucose uptake indicative of diminished neuronal activity/density, and the presence of dense senile plaques (SP) and neurofibrillary tangles (NFT), which contain aggregates of amyloid-β (Aβ) peptides and hyperphosphorylated tau (ptau) proteins, respectively.[3−7] As such, AD is classified as a protein misfolding disease. Currently, there is no cure for AD; present therapeutic strategies only alleviate or treat symptoms.[1,3,4,8] Although AD pathology is relatively well understood, disease etiology is still uncertain. A fundamental understanding of disease causing agents is necessary to develop diagnostics and therapeutics for preventing or curing AD. The volume of research in AD is vast due to the mounting urgency for a cure. In this review, we introduce in a tutorial format an overview of misfolded proteins (*i.e.*, Aβ, tau/ptau) and their potential involvement in neuropathogenesis of AD in the context of the amyloid cascade and tau hypotheses. Furthermore, the possible role of metal ions in Aβ and tau/ptau pathologies is presented, as well as Aβ- or ptau-mediated metal ion dyshomeostasis and miscompartmentalization. Due to the continued uncertainty in the root cause of AD, this review notably describes these three proposed factors (Aβ, tau/ptau, and metals) in equal measure and their inter-connections in AD onset and progression.

AMYLOID PRECURSOR PROTEIN (APP)

Aβ peptides are derived from APP, which is expressed in various tissues and organs of the human body, including the brain.[3−7] APP, which consists of three splice variants (APP_{695}, APP_{751}, and APP_{770}), is a type I membrane protein with one transmembrane domain (TMD) (Figure 1). The N-terminal domain resides in the extracellular space; the C-terminal

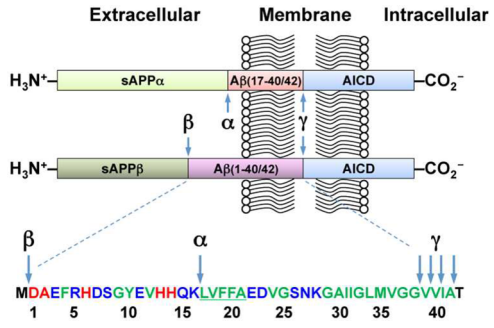


Figure 1. (Top) Schematic representation of APP and its cleavage by α-, β-, and γ-secretases. APP cleavage by α-secretase releases soluble fragment (sAPPα); subsequent cleavage by γ-secretase generates either Aβ(17−40) or Aβ(17−42) and AICD. Alternatively, if β- and γ-secretases perform the cleavage of APP, soluble sAPPβ and Aβ (mainly, Aβ(1−40/42)) are formed. (Bottom) Amino acid sequence of Aβ(1−42): black, flanking APP residues; red, putative Cu^{2+}-binding residues; blue, hydrophilic residues; green, hydrophobic residues; underlined, self-recognition region. The color code illustrates the bipolar nature of Aβ with a hydrophilic N-terminus and a hydrophobic C-terminus. Starting from the N-terminus, arrows indicate cleavage sites by β-, α-, and γ-secretases, respectively.

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domain, called APP intracellular domain (AICD), partially resides in the cytoplasm. APP is a metalloprotein whose function has not been fully elucidated; possible roles in metal ion homeostasis (e.g., copper (Cu) and iron (Fe)) and ferroxidase activity have been suggested.[6,9,10] Two putative metal binding sites of APP reside within the E1 (124−189, APP_{770} numbering) and E2 (376−554) domains with K_d values *ca.* 10^{-8} M for Cu^{2+} and *ca.* 10^{-6} M for Zn^{2+}.[6,9,11] There are no reports that the Aβ region of APP interacts with metal ions prior to enzymatic cleavage.

APP can be processed *via* amyloidogenic or nonamyloidogenic pathways, leading to different outcomes (Figure 1).[3−7] In the nonamyloidogenic route, APP is cleaved between Lys687 and Leu688 (APP_{770} numbering) by α-secretase, a multidomain zinc metalloenzyme, releasing the soluble N-terminal fragment sAPPα. The remaining membrane bound fragment is then cleaved by γ-secretase within the TMD of APP at either positions Val711/Ile712 or Ala713/Thr714 producing Aβ(17−40) or Aβ(17−42).[3−7] In the amyloidogenic pathway, the initial cleavage of APP is performed by β-secretase (BACE1), a copper metalloprotein. BACE1 cleaves between Met671 and Asp672, releasing the soluble fragment, sAPPβ. The remaining membrane-bound C-terminal fragment is then cleaved by γ-secretase, mainly generating amyloidogenic peptides Aβ(1−40) (~90%) and Aβ(1−42) (~10%) (Figure 1).[3,4,6] Both peptides are aggregation-prone and have a tendency to form oligomers and fibrils (Figure 2).

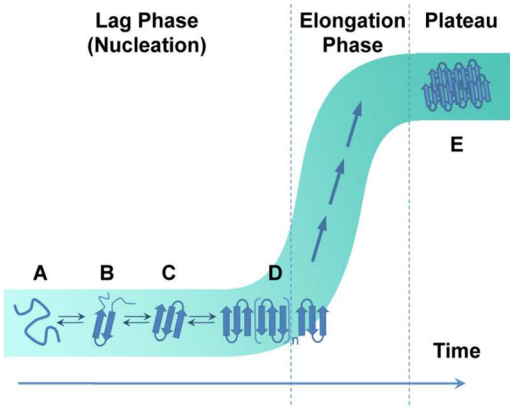

Figure 2. Scheme of "nucleated growth mechanism" represented as a sigmoidal curve to illustrate two-phase kinetics. There is an initial, slow nucleation phase when soluble, monomeric, or natively unfolded proteins (A) form β-structures (B) which aggregate to generate the initial nucleus (C) and protofibrils (D). This is followed by a fast elongation phase in which monomers and higher order oligomers elongate the nucleus into mature fibrils at the plateau (E). The aggregation kinetics and resultant fibril morphology are dependent on growth conditions (*e.g.*, pH, temperature, agitation); exogenous species such as metal ions, small molecules, and other proteins may also influence the rate of aggregation. This figure is adapted from ref 22 with permission.

AMYLOID-β (Aβ)

The amyloid cascade hypothesis suggests that aggregates of Aβ are the pathogenic agents in AD, initiating a cycle of harmful physiological changes that lead to neurodegeneration, dementia, and ultimately death.[3−7] Although insoluble Aβ fibril deposits in SP were thought to be the culprit, recent evidence indicates that Aβ oligomers are the toxic agent.[12,13] Experimental studies using soluble Aβ oligomers isolated from AD brains demonstrated that the oligomers could impair synapse plasticity and function (*vide infra*).[12,13] Amyloid fibrils may still be harmful, possibly acting as a "reservoir" for oligomers, where an equilibrium is established between insoluble Aβ fibrils and soluble, toxic Aβ oligomers.[3] Therefore, AD can be classified as a "gain-of-toxic function" disease associated with the toxicity of aggregated Aβ species.

Both monomeric peptides, Aβ(1−40) and Aβ(1−42), adopt mostly unfolded random coil conformation with some α-helical and β-sheet content. The peptide sequence is bipolar, with a hydrophilic N-terminus and a hydrophobic C-terminus (Figure 1).[4−7,14] The C-terminus and central hydrophobic residues Leu17−Ala21 are believed to be essential for Aβ aggregation. The C-terminus also facilitates the interaction of Aβ with membranes, possibly enhancing Aβ aggregation. Plaques of aggregated Aβ are enriched in Aβ(1−42) relative to Aβ(1−40).[3−7] Aβ(1−42) is more aggregation-prone than Aβ(1−40), possibly due to the additional two hydrophobic C-terminal residues. Aβ(1−42) is thought to be more pathogenic since it may form toxic oligomers more readily than Aβ(1−40).[3−7]

Aggregated Aβ forms (*e.g.*, oligomers, fibrils) have inherent β-sheet structures as determined by solid-state nuclear magnetic resonance (ssNMR) spectroscopy.[15−20] Individual soluble monomers first form β-structures as seeds.[21−23] β-Structures from different peptides then generate intermolecular hydrogen bonds and hydrophobic contacts, forming higher order aggregates. Spherical particles, 3−10 nm in diameter, have been imaged by atomic force microscopy (AFM) before their arrangement into other structures, such as annular-protofibrils that have a pore within a β-barrel structure.[24] Higher order structures, including protofibrils,[25] globulomers,[26,27] Aβ-derived diffusible ligands (ADDLs),[28] amylospheroids,[29,30] and tubular oligomers,[16] have also been observed. Different Aβ oligomers have been associated with diverse mechanisms of toxicity. The smallest neurotoxic oligomers identified are dimers, and larger aggregates, such as trimers, hexamers, and dodecamers, have also exhibited neurotoxicity.[31−33] Aβ oligomers are suggested to insert into membranes and behave like cation-selective ion channels allowing unregulated entry of Ca^{2+} into neurons.[34] Membrane insertion of Aβ oligomers could push phospholipid head groups apart, disrupting membrane conductance and cation/anion homeostasis.[35] Since neuron transmission is dependent on maintaining a certain membrane potential, altered membrane conductance could result in the breakdown of signal transduction and cytotoxicity from unregulated metals. Another mechanism of Aβ oligomer toxicity is *via* binding to synaptic protein receptors, such as by ADDLs, which weakens neuronal plasticity and consequently impairs memory.[36] Although Aβ oligomers have been suggested to exert cytotoxicity, many studies are performed on *in vitro* prepared oligomers,[24−27,29,31] with fewer oligomers isolated *ex vivo* from AD brain tissue.[12,13,30] It remains to be determined how relevant *in vitro* prepared oligomers are, yet they can still provide insight into Aβ oligomers' involvement in AD pathogenesis. Oligomers isolated from AD brain tissue[12,13,30] may have undergone transformation from the original species present *in vivo* during isolation processes. Therefore, there is uncertainty in the nature of *in vivo* oligomers and the mechanisms by which they could be associated with toxicity. Undiscovered types of Aβ oligomers could also be linked to neurotoxicity in AD. Despite differing modes of action, Aβ oligomers could be involved early in AD, prior to onset of

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neurodegeneration, and may explain why disease severity does not always correlate with plaque load. It has been suggested that insoluble fibrils may sequester soluble oligomers as a neuroprotective measure.[3]

Aβ fibril formation occurs *via* a "nucleated growth mechanism" (Figure 2) that is initiated by a lag phase and followed by an elongation phase, culminating in mature fibril formation.[21,22,37] During the relatively slow nucleation phase, natively unfolded Aβ forms nuclei for further growth into fibrils. Elongation occurs rapidly once the lag phase is surmounted.[16] The time trace for this two-phase process is sigmoidal (Figure 2) and has been monitored by thioflavin-T (ThT) fluorescence, molecular tumbling anisotropy, circular dichroism (CD) spectroscopy, Fourier transform infrared (FTIR) spectroscopy, and dynamic light scattering.[7] The morphology of resultant fibrils is dependent on a broad spectrum of initial conditions such as temperature, pH, presence of exogenous species, and agitation.[16,21,22] The diverse morphology also results from heterogeneity and flexibility in monomer conformation, which is influenced by solution conditions. Initiation and aggregation rates are decided by a variety of factors: (1) natural propensity of a particular peptide sequence to aggregate; (2) solution pH; (3) peptide concentration; (4) presence/absence of exogenous species (*e.g.*, metal ions, other proteins).

Aβ fibrils adopt a cross-β structure in which β-strands from aggregating peptides are perpendicular to the direction of fibril elongation.[16,21,22] Fibril structural information has been gathered from ssNMR and site-directed spin labeling electron paramagnetic resonance (SDSL-EPR) spectroscopy in conjunction with gross morphology imaged by transmission electron microscopy (TEM), AFM, and X-ray diffraction. Aβ(1−40) fibrils grown with agitation have striated-ribbon morphology whereas fibrils generated under quiescent conditions have a twisted-pair morphology.[15,16,18] The propagating unit in striated-ribbon is composed of two cross-β units arranged around a pseudo C2 axis coincident with the fibril axis (Figure 3A), while a twisted-pair propagating unit contains three cross-β units arranged around a pseudo C3 axis (Figure 3B).[15,18,38] The fibrillar structure of Aβ(1−42) has also been investigated; its cross-β units adopt a structure containing β-strands and a β-turn, similar to those in Aβ(1−40), but spanning different residues.[19] Fibrils seeded from Aβ fibrils isolated from human AD brain tissue demonstrate that they might adopt a different morphology to either striated ribbon or twisted pair,[39] and these Aβ fibrils may have more relevance to AD pathology *in vivo*. Advances in techniques may help shed more light on structural information of *in vivo* fibrils in detail.

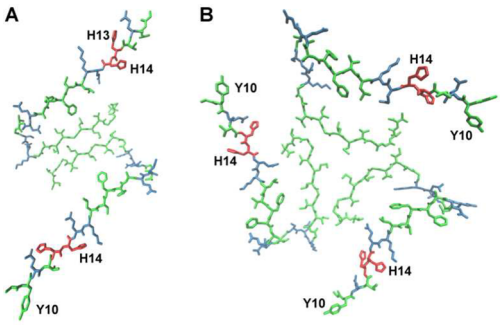

Figure 3. Propagating units from Aβ fibrils grown (A) with agitation resulting in striated-ribbon morphology (PDB 2LMN),[15] shown down the pseudo C2 axis, and (B) without agitation resulting in twisted-pair morphology (PDB 2LMP),[18] shown down the pseudo C3 axis. Cross-β units contain β-strands (residues 10−22 and 30−40), a β-turn (residues 23−29), and a disordered region (residues 1−9 which are not displayed in the figure). Importantly, striated-ribbon cross-β units have a salt bridge between Asp23 and Lys28 that stabilizes the β-turn and, therefore, the entire β-hairpin structure, promoting nucleation for fibril growth.[15,20] The color code is the same as in Figure 1: putative Cu^{2+}-binding residues (red); hydrophilic residues (blue); hydrophobic residues (green). Figures are depicted using Visual Molecular Dynamics (VMD): http://www.ks.uiuc.edu/Research/vmd/.[38]

TAU

Another hallmark of AD is the deposition of NFT, composed mainly of misfolded hyperphosphorylated tau (ptau) aggregates, which leads to consideration of the tau hypothesis to describe ptau toxicity and the accompanying physiological changes resulting in neuropathogenesis.[3,40−43] Tau is a member of the microtubule-associated protein (MAP) family, which imparts structural integrity and stability to microtubules. Microtubules act as dendrite scaffolding, maintaining neuron shape and hence the contact between neurons for signal transduction as well as memory processing and retention. In addition, tau assists in anterograde (nucleus to periphery) and retrograde (periphery to nucleus) shuttling of essential nutrients, neurotransmitters, and organelles along the axon, which is necessary for neuronal plasticity.[3,40−43]

Tau exists in six isoforms varying in length from 352 to 441 residues. These isoforms differ in the numbers of highly conserved microtubule-binding repeats (R) in the microtubule-binding domain (3 or 4 near the C-terminus) and acidic amino acid stretches (N) (0, 1, or 2 near the N-terminus) (Figure 4A).[40−43] In addition, all isoforms contain a proline-rich domain between these two regions. Tau is water-soluble due to its large number of charged residues.[42,43] The range of net isoelectric point (pI) is 6.5−9.5 for different isoforms. The N-terminus is negatively charged at physiological pH, whereas both proline-rich domain and microtubule binding domain are positively charged at pH 7.4 (Figure 4B).[42,43] The binding model between tau and microtubules suggests a finely honed

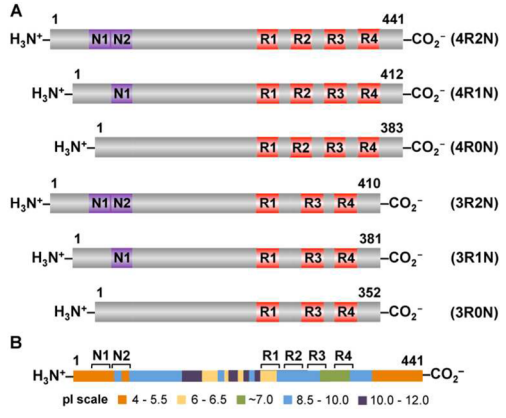

Figure 4. Tau isoforms and pI distribution of tau_{441} isoform. (A) Six tau isoforms. Purple bands indicate acidic amino acid stretches (N) near the N-terminus. Red bands indicate microtubule-binding repeats (R1, R2, R3, and R4) at the C-terminus. Combination of three or four microtubule-binding repeats in the absence or presence of zero, one, or two acidic amino acid stretches generates six tau isoforms varying in length from 352 to 441 amino acids. (B) The calculated pI distribution of tau_{441} isoform (4R2N).[40,42,43]

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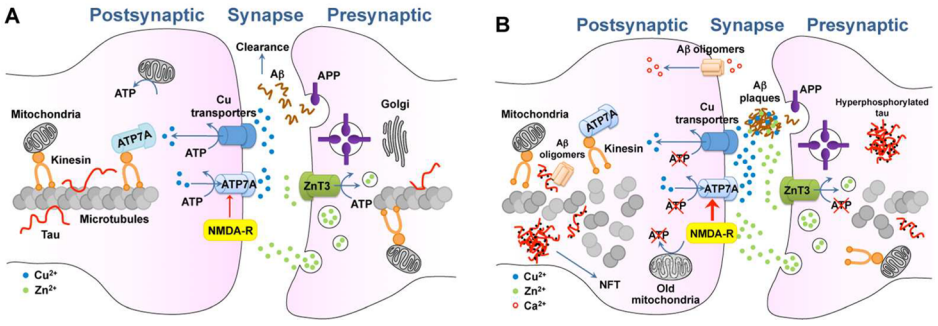

Figure 5. Schematic representation of Aβ and tau pathologies and metal ion dyshomeostasis in AD brains. (A) Proposed normal synaptic function. Zn^{2+} transporter, ZnT3 (green), loads Zn^{2+} (green spheres) into vesicles for release upon signal transduction. Zn^{2+} binds to NMDA receptors (NMDA-R, yellow) at the postsynaptic terminal, stimulating Cu^{2+} release (blue spheres) into the synapse *via* the copper transporter ATP7A (light blue). Reuptake of copper post neuron firing is by an as yet unidentified copper transporter (dark blue), probably in an energy-dependent manner. Simultaneously, Aβ (brown), from proteolytic APP processing (purple), can be cleared *via* various mechanisms, and tau (red)-stabilized microtubules (ordered gray spheres) support axonal transport of new organelles to the terminus. (B) Suggested synaptic dysfunction, which could result from a lack of ATP, triggering lowered release and reuptake of metal ions. Decreased neuronal concentrations of zinc and copper could also decrease the activity of zinc-dependent Aβ degrading enzymes, resulting in elevated amounts of Aβ oligomers and fibrils. Aβ oligomers (light orange) could bind to tau, causing it to detach from microtubules and leading to depolymerization and stalling of anterograde axonal transport. Aβ oligomers could also cause tau hyperphosphorylation (black spheres), inducing its detachment from microtubule and aggregation into NFT, further obstructing axonal transport. Aβ oligomers could also insert into the axon membrane and affect Ca^{2+} transport into the neuron, further destabilizing neuron function.

interaction; the microtubule binding repeats of tau interact with β-tubulin on the inner microtubule surface, whereas the positively charged prolines on tau interact with negatively charged residues on the microtubule surface.[40] The binding of tau to microtubules is modulated by kinases and phosphatases, which alters tau's charge distribution.[40−43] Phosphorylation of tau detaches it from microtubules, removing the barrier to dynein-mediated anterograde and kinesin-mediated retrograde transport; dephosphorylation helps it associate to microtubules, placing a barrier to vesicular transport.[40] Thus, tau is able to regulate axonal trafficking.

HYPERPHOSPHORYLATED TAU (PTAU)

In AD, an imbalance between kinase and phosphatase activities results in accumulation and aggregation of chronically hyperphosphorylated tau (ptau).[40,42−44] Although the cause and mechanism for this imbalance are still unclear, possible factors such as Aβ (*vide infra*), age, genetics, and environment have been suggested.[42,44] Since ptau pathology occurs in other neurodegenerative tauopathies, biochemical factors apart from Aβ may also be involved in initiating disease according to tau hypothesis. Kinases can phosphorylate Ser, Thr, and Tyr residues at their hydroxyl groups. The longest tau isoform has 85 such residues, 71 of which have been identified as possible phosphorylation sites in physiological or pathological tau. Most phosphorylation sites flank the microtubule-binding domain.[43,45] A group of 20 kinases is known to participate in tau phosphorylation at various positions.[42,43] The major phosphatase responsible for ptau dephosphorylation is phosphatase-2A (PP2A), whose inhibitors are overexpressed in AD. Treatment of ptau with PP2A could restore tau activity.[46]

The highly conserved C-terminal repeats (R2 and R3 in 4R tau; R3 in 3R tau; Figure 4A) are the most aggregation-prone regions of tau.[42,43] In normal tau, both N-terminal and C-terminal flanking regions of the microtubule-binding domain are positively charged and inhibit tau aggregation (Figure 4B).[47] Hyperphosphorylation of tau significantly alters its pI, thus facilitating its aggregation into oligomers and amorphous tangles that mature into paired helical filaments (PHF) in AD.[41,42] In NFT, PHF are co-precipitated with other proteins including various post-translationally modified and truncated tau/ptau proteins.[40,41,48] Amyloid-like cross-β features have been indicated for *in vitro* grown recombinant PHF as well as PHF isolated from Down syndrome brains.[49] Despite significant advances, *in vitro* grown PHF may not be representative of PHF from AD brains because it is challenging to mimic growth conditions in the brain and interpretation is complicated by tau's phosphorylation state. PHF were initially suspected of being the toxic species, but recent evidence suggests that the soluble prefibrillar forms of ptau are the toxic species.[42,44,48] For example, *Drosophila* overexpressing tau exhibited signs of neurodegeneration prior to NFT formation,[50] which was also observed in mouse models.[51]

Since tau has a natural function, ptau pathology can be characterized by a loss-of-normal function and/or gain-of-toxic function disease.[40−42,44,48] Compared to tau, ptau has a lower affinity for microtubules.[40] Therefore, it does not bind to microtubules, which leads to microtubule depolymerization, disruption in axonal transport, and loss of dendrite structure.[40,41,48] pTau is more resistant to proteases than tau and thus can accumulate,[52] sequestering normal tau and other MAPs (*e.g.*, MAP1/2), which results in further disturbance to microtubule function.[53] The combined effect starves dendrites from the contents of cargo vesicles: nutrients, neurotransmitters, growth factors, and whole organelles such as mitochondria and peroxisomes. The neuron's response to nutrient deprivation is autophagy, but autophagosomes in AD brains do not fuse with lysosomes for recycling of cell contents.[3,54] Upon AD progression, the number of stalled autophagosomes increases, inducing neuron swelling and inflammation. Furthermore, if new organelles do not reach dendrites due to collapse of axonal transport, old organelles, such as spent mitochondrias, cannot be replaced.[3,54] They would continue to function at a diminished capacity, unable to meet the neuron's energy demand or to scavenge ROS that leak into the cytoplasm and cause oxidative stress (Figure 5). pTau oligomerization may enhance its natural ability to bind membranes *via* its N-

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terminus, which could possibly disrupt the membrane potential[55] and other related functions in a manner similar to Aβ oligomers (*vide supra*).[44] Indeed, C-terminal truncated ptau tetramers were shown to diminish Ca^{2+} buffering in immortalized cortical cells with concurrent loss of mitochondrial membrane potential.[44] Tau binding partners have not been fully identified, and aberrant interaction of tau with them may result in further loss-of-normal function or gain-of-toxic function.[40]

Aβ AND TAU/PTAU

The amyloid cascade hypothesis has been a prevailing explanation for AD pathology for two decades, and there are several alternative explanations, such as the tau hypothesis. Thus, numerous drugs in development target Aβ pathology,[3,4,8] as well as some promising candidates for ptau pathology.[40,48] Despite the prominence of the amyloid cascade hypothesis, there are aspects of AD it cannot explain. For example, the extent of neurodegeneration correlates with the amount of NFT but not with Aβ plaque load.[3,6,42] The discrepancy might be explained if soluble Aβ oligomers are the toxic species, or if Aβ influences ptau pathology and therefore NFT load. The relationship between these two contributors to AD is still uncertain and is an active field of research seeking to establish which pathogenesis occurs upstream.[56−60] Current data implicates Aβ-mediated pathology as the upstream event[59,60] with ptau as an active and necessary participant in neurodegeneration.[56,57] pTau pathology exists in various neurodegenerative tauopathies in the absence of Aβ and thus may arise through independent biochemical pathways.[42]

It has been shown that Aβ and ptau pathologies are intertwined either directly or indirectly. A direct interaction suggests contact between Aβ and tau through protein−peptide complex formation, which may alter tau properties, leading to expression of pathogenicity. Such Aβ−tau complexes have been detected by Western blotting and enzyme-linked immunosorbent assay (ELISA).[58] Complexation enhanced tau as a substrate for glycogen synthase kinase 3β, a kinase implicated in pathogenic ptau production. Tau phosphorylation resulted in complex disassembly and ptau dissociation from microtubules, leading to breakdown of axonal transport. Indirect mechanisms of action exist where Aβ has been shown to upregulate kinases and proinflammatory cytokines that modulate tau phosphorylation and also to inhibit ptau degradation.[61] Aβ oligomers (*e.g.*, dimers and tetramers of Aβ(1−42);[59] 8−24 mers of Aβ(1−40)[59,60]) have been tested and shown to affect tau pathology,[59,60] although an earlier experiment suggested Aβ fibrils instead.[62] Addition of oligomeric Aβ to tau expressing non-neuronal cells resulted in tau-dependent loss of microtubule structure.[59] Cells were particularly susceptible to Aβ(1−42) oligomers, as these oligomers inhibited microtubule function at concentrations significantly lower than concentrations of Aβ(1−40) oligomers. pTau truncation in cortical neurons occurs upon treatment with Aβ and may be another means by which Aβ affects ptau pathology.[63] pTau truncated at D421 has been shown to possibly disrupt Ca^{2+} homeostasis and mitochondrial function. Although Aβ currently appears to be the upstream event, Aβ and ptau exert their influence together through deprivation of axonal transport leading to neuronal degeneration and behavioral deficit.[56−60] A better understanding of their relationship could be beneficial for elucidating AD pathogenesis, especially how they interact directly at a molecular level, and whether modulation of their interaction might ameliorate tau/ptau dependent deterioration.

NEUROBIOLOGY OF METAL IONS

Copper, zinc, and iron are essential metals for healthy organisms and brain function.[6,64−66] Cu is transported in the plasma by copper storage proteins (*e.g.*, ceruloplasmin, albumin) and delivered across the blood brain barrier (BBB) and cell membranes by copper transporters (*e.g.*, ATP7A, Ctr1).[9,64−66] Zinc is delivered by metallothioneins and penerates the BBB and membranes *via* transporters (ZnT1−ZnT10).[6,66,67] Iron is transported by ferritin, which traverses the BBB and cell membranes *via* transferrin receptor-mediator endocytosis.[66]

Copper and zinc are observed to be involved in neuronal pathways in the brain.[6,64−67] During normal signaling, zinc transporter, ZnT3, loads Zn^{2+} into vesicles that are released from the presynaptic neuron with glutamate neurotransmitters (Figure 5A).[6,66,67] High concentrations of Zn^{2+} flood the synapse, attaining concentrations as high as 300 μM.[6,66] This extracellular Zn^{2+} binds to *N*-methyl-D-aspartic acid receptors (NMDA-R), activating the Cu transporter ATP7A, which releases Cu^{2+} into the synapse (*ca.* 15−30 μM) (Figure 5A).[6,9,65,66] Reuptake of metal ions into the pre- and postsynaptic neurons occurs against a concentration gradient and is therefore probably energy-dependent.[6,9,65−67] Alternatively, metallothionein MT3 also binds Cu^{2+} and Zn^{2+} for reuptake.[6,9,64,66]

Homeostasis and functions of metal ions are highly regulated in living organisms; therefore, dysregulation of metal ions can initiate and progress pathological pathways resulting in disease or death.[6,66] In AD, the accumulation of misfolded Aβ and ptau aggregates are accompanied by metal ion dyshomeostasis and miscompartmentalization.[6,10,68] Highly concentrated copper (*ca.* 0.4 mM), zinc (*ca.* 1 mM), and iron (*ca.* 0.9 mM) have been detected in SP of AD brains.[6,68,69] In addition, NFT may also be associated with high levels of redox active iron and copper.[70] Details of metal binding to Aβ and tau/ptau is discussed in the following sections.

Aβ−METAL BINDING

Metal binding facilitates Aβ nucleation, increases peptide aggregation rate, and generates different conformations of Aβ aggregates (e.g., nonfibrillar forms dependent on metal:Aβ stoichiometry).[6,69,71−73] Metal binding to Aβ species has been extensively studied through physical methods.[9,73−80] The coordination environment of metal ions to Aβ depends on experimental conditions (*e.g.*, pH, buffer). Aβ binds Cu^{2+} *in vitro* with K_d ranging from *ca.* 10^{-11} to 10^{-7} M for 1:1 Aβ−Cu^{2+} complexes.[6,69,71−73] Cu^{+} also forms 1:1 complexes with Aβ(1−16) and Aβ(1−42) with K_d values of *ca.* 10^{-14} and *ca.* 10^{-7} M, respectively.[77] Zn^{2+} binds to Aβ with K_d values ranging from *ca.* 10^{-9} to 10^{-6} M.[6,69,71,72] Fe^{2+} forms complexes with Aβ(1−16) (K_d, *ca.* 10^{-4} M, which suggests a weaker binding of Fe^{2+} to Aβ compared to Cu^{2+} and Zn^{2+}).[78,80]

The primary coordination sphere of Aβ−Cu^{2+} is dynamic and pH dependent; resulting complexes exist in two forms, called component I and II.[68,71−73] At physiological pH, component I is predominant, with three nitrogen and one oxygen (3N1O) donor atoms from Aβ coordinating to Cu^{2+}.[6,68,69,71,73] The proposed donor atoms are derived from either three His residues (His6, His13, and His14) with a Asp1

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carboxylate or a backbone carbonyl between Asp1 and Ala2 (Asp1−Ala2), or from two His residues, an N-terminal amine, and a Asp1−Ala2 backbone carbonyl, possibly with Asp1 carboxylate.[6,68,69,71,73] Above *ca.* pH 8, component II is prevalent, with a different 3N1O coordination environment of Cu^{2+}, composed of either three His residues and the Ala2 main chain carbonyl or a mixture of N-terminal amine, a deprotonated Asp1−Ala2 backbone amide, a Ala2−Glu3 backbone carbonyl, and a His residue.[6,68,69,71,73] In addition, a 4N coordination mode has also been suggested for component II, generated from three His residues with either N-terminal amine or deprotonated backbone amide.[6,68,69,71,73] In the case of Cu^{+}, extended X-ray absorption fine structure measurements propose a bis-His linear complex of Cu^{+} with Aβ(1−16) and Aβ(1−42) monomers.[74−77] NMR results indicate that Cu^{+} binding with Aβ occurs *via* the histidine residues His6, His13, and His14, but only two His residues bind at any one time.[79] The coordination mode of Aβ−Zn^{2+} involves 4−6 ligands.[68,69,71−73] There is an agreement that all three His residues bind with additional candidates being Asp1 (through either N-terminal amine or side chain carboxylate), Arg5 (backbone amide), Tyr10, Glu11, or water molecules.

Addition of Cu^{2+} *in vitro* has been shown to enhance the proportion of β-sheet content in both Aβ(1−40) and Aβ(1−42).[81,82] Since nucleus formation is believed to involve β-structure formation, the increased proportion of β-sheet in copper-bound Aβ likely indicates facilitation of nucleation and propensity to aggregate. This is in accordance with numerous studies that have presented accelerated rates of Aβ aggregation in the presence of certain divalent metal cations (*e.g.*, Cu^{2+}, Zn^{2+}).[10,72,83] As well as influencing the aggregation rate of Aβ, metal ions generate more amorphous aggregates and/or potentially neurotoxic oligomers.[81,84] In addition, Aβ−$Cu^{2+/1+}$ could participate in ROS production and Fenton-like chemistry causing oxidative stress and neuronal damage (*vide infra*).[6,9] Despite their proposed importance, little is known on the mechanistic details of Aβ−metal aggregation and, to the best of our knowledge, Aβ−metal oligomers have not been isolated to date.

Aβ-METAL-MEDIATED OXIDATIVE STRESS

Redox active metals (*e.g.*, copper, iron) associated with Aβ species have been implicated in pathological pathways, such as ROS formation (*e.g.*, hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet-}$)) leading to oxidative stress.[6,85] *In vitro* experiments on Aβ-$Cu^{2+/1+}$ suggests that reduced Aβ−Cu^{+} is accessible to common exogenous cellular reductants such as ascorbate and glutathione.[86,87] Experiments have demonstrated the ability of Aβ−$Cu^{2+/1+}$ to catalytically produce H_2O_2 and participate in Fenton-like hydroxyl radical (•OH) production using ascorbate as a reductant. When Aβ−Cu^{2+} is reduced to Aβ−Cu^{+}, there are multiple avenues for reaction (Figure 6).[5,6,85] The first is a catalytic cycle, called the H_2O_2 cycle, which begins by O_2 binding to Aβ−Cu^{+}, followed by electron transfer from Cu^{+} to O_2, forming $O_2^{\bullet-}$. Proton-coupled electron transfer generates a hydroperoxy radical ($HO_2^{\bullet-}$), which accepts a proton (H^{+}), affording H_2O_2. The H_2O_2 cycle is only catalytic with respect to Aβ−Cu^{2+}; exogenous reductant is used quantitatively, making the cycle doubly damaging to cells. In addition to production of H_2O_2, the cell's supply of antioxidants is depleted. In tandem with the H_2O_2 cycle is the Fenton pathway (Figure 6). Under a low O_2 pressure, or in the presence of increasing H_2O_2 concentration from the H_2O_2 cycle, Aβ−Cu^{+} can bind H_2O_2 instead of O_2, leading to Fenton-like chemistry and formation of •OH. Another pathway may compete with the H_2O_2 cycle, where $O_2^{\bullet-}$ can dissociate from the intermediate Aβ−Cu^{2+}-$O_2^{\bullet-}$ (Figure 6).[5,6,9,85] If $O_2^{\bullet-}$ is not scavenged by superoxide dismutase, it can react with nitric oxide (NO) to form peroxynitrite (ONO_2^{-}), which causes lipid peroxidation, protein oxidation, protein nitration, and DNA oxidation. Aside from participating in the Fenton pathway, H_2O_2 is itself a strong oxidant that can form peroxy adducts of organic molecules. It is also employed naturally in cells as a signaling molecule in inflammatory responses and has been shown to upregulate stress-activated protein kinases and tau phosphorylation proteins in AD.[3] ROS production by Aβ−$Cu^{2+/1+}$ has been studied *in vitro* and/or *in silico*, as has Aβ oxidation/cross-linking at/*via* methionine or tyrosine.[3,5,9] It has been challenging to establish a reduction potential for various forms of Aβ *in vitro* and *in vivo*; therefore, reactions that are disfavored *in vitro* may still occur in diseased settings.

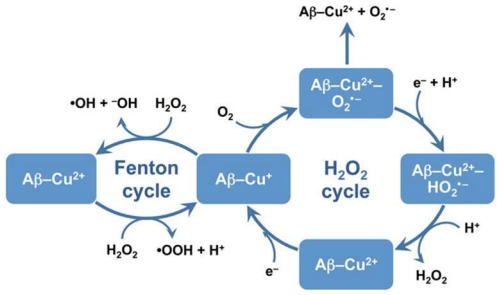

Figure 6. Proposed ROS production by Aβ−Cu^{2+}. Central, catalytic hydrogen peroxide (H_2O_2) cycle with superoxide ($O_2^{\bullet-}$) dissociation pathway (right) and Fenton cycle (left).

TAU-METAL BINDING

There are relatively fewer studies of metal binding to tau/ptau compared to those of Aβ.[88−93] Cu^{2+} binding to various tau fragments containing different microtubule-binding repeats (R) has been demonstrated *in vitro* by mass spectrometry, CD, and NMR.[89−91] The third repeat, R3, binds more than one Cu^{2+} equivalent through two His residues. CD measurements demonstrate a β-sheet enriched transition upon addition of one Cu^{2+} equivalent.[89] Similar Cu^{2+}-binding studies for R1 and R2 demonstrated some Cu^{2+} binding and alteration in the secondary structure.[90,91] Cu^{2+} binding to full length tau and to a construct containing only four repeats (called K32) showed 1:1 binding stoichiometry, suggesting that Cu^{2+} was bound specifically to the microtubule-binding domain, probably *via* two Cys residues.[88] Cu^{2+} addition to K32, however, did not influence secondary structure, and only limited aggregation was observed. Trivalent cations, such as Fe^{3+} and Al^{3+}, have been shown to induce ptau aggregation.[92,93] Ascorbate-mediated reduction of Fe^{3+} to Fe^{2+} reversed aggregation.[92] Overall, tau and ptau may interact with metal ions, but the full extent has not been investigated. Studies on truncated and/or unphosphorylated tau have been reported to date, but this may not mirror ptau−metal interactions *in vivo*. Phosphorylation introduces negative charge(s), which favors an electrostatic interaction with metal ions. NFT have been shown to contain metals,[70] which suggests the possibility of ptau binding to metal ions. Further clarification of metal interaction with tau/ptau warrants consideration, with particular attention to tau's phosphorylation state.

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Although tau−metal-mediated oxidative stress has not been as extensively addressed as for Aβ (*vide supra*), it has been shown that incubation of R2 fragment from tau with Cu^{2+} leads to H_2O_2 production.[94] Cu^{2+} was reduced to Cu^{+} with concomitant intermolecular peptide cross-linking through cysteine disulfide bond formation of the oxidized peptide.[94] *In vitro* studies on hippocampal tissue slices also demonstrated ROS production in both SP and NFT.[70] Reports on tau−metal-mediated oxidative stress are scarce because metal binding studies are limited, but this may be an area worthy of closer scrutiny.

METAL ION DYSHOMEOSTASIS IN AD

In constrast to the distribution of copper and zinc found in normal neuronal pathways (*vide supra*), in the energy starved AD brain, Zn^{2+} reuptake after neuronal signaling is slowed; thus, Zn^{2+} activation of NMDA receptors (NMDA-R) is prolonged, which releases more Cu^{2+} into the synapse whose reuptake is also inefficient (Figure 5B).[3,6,64,66] As a result, higher concentrations of metal ions could accumulate within the synapse, which could seed Aβ aggregation.[3,6,64,66] Once aggregation is initiated, these metal ions are sequestered within Aβ fibrils, becoming relatively deficient in other areas of the neuron, leading to miscompartmentalization. AD mouse models lacking ZnT3 gene cannot load Zn^{2+} into vesicles for presynaptic release; therefore, they would not build a constant pool of Zn^{2+} within their synapses. Their brains showed 50% less plaque formation than those from AD models expressing ZnT3 normally, suggesting a possible role of Zn^{2+} in seeding Aβ aggregation.[95] Although metal ion miscompartmentalization is evident since Aβ plaques are shown to contain high concentrations of metals, the overall level of metal ions is uncertain in AD brains compared to those in healthy brains.[6,10,64] The heterogeneity of brain samples could be responsible for the inconsistency in reports.[6,10,64] A greater understanding of dyregulated metals in AD could help in the design of diagnostics and therapeutics.

In addition to Aβ, tau pathology can also perturb metal ion homeostasis in the brain by several mechanisms (Figure 5B).[3,42,56,57,70] One mechanism is through degradation of axonal transport which consequently disrupts anterograde vesicular movement. Vesicles would no longer arrive at the axon terminal, and their cargo contents such as metal ion transporters would not be deployed to the synapse. The copper transporter ATP7A has been observed to translocate from the Golgi to the cell periphery *via* microtubules.[96] ATP7A transports copper into the brain across the blood−brain barrier and provides neurons with a source of copper for insertion into metalloenzymes. Therefore, disruption of axonal ATP7A transport could have a downstream effect on metal ion homeostasis. Another downstream effect is from the transport of new organelles such as mitochondria from the cell body to the periphery, which also translocate *via* microtubules.[40,57] If new mitochondria do not replace old ones, the axon terminal cannot meet its energy demands and reuptake of Cu^{2+} and Zn^{2+} from the postsynaptic neuron firing is slowed. This triggers chronically elevated levels of the two metal ions in the synapse (*vide supra*) (Figure 5B). Finally, another disruption of metal ion homeostasis by tau is sequestration of metal ions within NFT.[70] It has been established that NFT contain noticeable levels of redox active metals, leading to metal ion dyshomeostasis and miscompartmentalization.

Furthermore, metal ions can influence Aβ and tau pathologies. Aβ production is regulated by zinc- (α-secretase) and copper-dependent (β-secretase) enzymes that will not be properly metalated if metal ions are dysregulated. In healthy brains, mechanisms exist for Aβ clearance to eliminate the peptide and thus prevent it from aggregating. For example, some proteases responsible for Aβ degradation are zinc-dependent neprilysin (NEP) and insulin degrading enzyme (IDE).[3] Metal ion dyshomeostasis could result in improperly metalated NEP and IDE, disrupting Aβ digestion, which would impact the rate of Aβ production versus clearance, exacerbating the formation of toxic aggregates. Likewise, tau phosphorylation could be influenced by zinc,[97] copper,[98,99] and iron,[100] which have been shown to up- or down-regulate kinases that phosphorylate tau, further worsening tau pathology.

This interplay among Aβ, tau, and metal ion dysregulation underlies a cascade effect in which all participants involved influence one another in a complex scenario and together have an aggravating effect on disease progression. Our understanding of Aβ, tau, and metal ions in AD is limited by the currently available data, but it is important to investigate them in greater detail in order to develop an innovative, multifaceted approach for AD treatment.

CONCLUSION

AD is a complex disease with multiple, intertwining pathological factors, making the elucidation of precise disease etiology challenging. In AD brains, accumulation of misfolded Aβ peptides and ptau proteins are observed. It is still unclear, however, if and how these misfolded aggregates are involved in the onset and progression of disease. The current amyloid hypothesis suggests that aggregated Aβ forms are the neurotoxic agents in AD; however, mechanisms leading to the imbalance in Aβ production versus clearance have not been fully revealed. In addition to Aβ, ptau could disassemble microtubules and interrupt intraneuronal trafficking of microtubule-dependent cargos. Furthermore, Aβ has been shown to affect tau pathology by triggering tau phosphorylation, proposing their potential inter-connection in AD neuropathogenesis. Another hallmark of AD is metal ion dyshomeostasis and miscompartmentalization. Metal binding to Aβ and tau is observed to facilitate aggregation, as well as generate ROS leading to oxidative stress and ultimately neuronal death. Aβ and tau pathologies may directly or indirectly prompt metal ion imbalance in the brain. Overall, current evidence suggests that Aβ, tau/ptau, and metal ions could be associated individually and/or mutually with AD pathogenesis. Gaining a greater understanding of their roles in AD will lead to a unified picture of disease etiology and contribute to the development of effective diagnostics and therapeutics.

AUTHOR INFORMATION

Corresponding Author

*E-mail: mhlim@umich.edu.

Present Address

[§]Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada.

Notes

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■ ABBREVIATIONS

Aβ, amyloid-β peptide; AD, Alzheimer's disease; ADDLs, Aβ derived diffusible ligands; AFM, atomic force microscopy; AICD, amyloid precursor protein intracellular domain; APP, amyloid precursor protein; ATP7A, ATPase copper transporting alpha peptide; CD, circular dichroism spectroscopy; EOAD, early onset Alzheimer's disease; FTIR, Fourier transform infrared spectroscopy; K_d, dissociation constant; LOAD, late onset Alzheimer's disease; MAP, microtubule-associated protein; NFT, neurofibrillary tangles; NMDA, *N*-methyl-D-aspartic acid; NMR, nuclear magnetic resonance spectroscopy; PHF, paired helical filaments; pI, isoelectric point; ptau, hyperphosphorylated tau; ROS, reactive oxygen species; SDSL-EPR, site-directed spin labeling electron paramagnetic resonance spectroscopy; SP, senile plaques; ssNMR, solid-state nuclear magnetic resonance spectroscopy; ThT, thioflavin-T; TMD, transmembrane domain; ZnT3, zinc transporter-3

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