Tau protein and the development of its visualization tools

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Abstract: Tau is a microtubule-associated protein (MAPT) that is highly expressed in neurons and participates in multiple cellular processes. Tau misfolding and self-aggregation produce protein deposits called neurofibrillary tangles. The pathological pathway from soluble monomer to insoluble filamentous Tau is considered to be the basis of human Tauopathies. Cases of frontotemporal dementia are caused by a dominant genetic mutation of the Tau gene MAPT. Advanced imaging methods have elucidated important structural and functional aspects of tau, and can be used as a diagnostic tool in clinical research. This review will focus on the latest developments in super-resolution imaging methods, near-infrared fluorescent probes, and positron emission tomography.

1. Introduction

1.1. Structure and function of Tau protein

Many neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, prion disease, etc. have a common pathological feature, that is, due to the accumulation of the microtubule-associated protein tau, leading to the abnormal deposition of neurofibrillary (NFTs) in the brain, and then produces neurodegenerative diseases. Such diseases are collectively referred to as tauopathy. Hyperphosphorylation of tau protein in the brain of AD patients reduces its ability to bind to microtubules. The phosphorylated tau protein aggregates to form intracellular neurofibrillary tangles, which interferes with the normal function of neurons, and ultimately leads to neuronal degeneration and death.

The Tau protein gene is mainly located on chromosome 17 and is encoded by a single gene. Its primary structural features are divided into four functional regions from carboxyl end to amino terminus: N-terminal projection domain, proline-rich domain, microtubule binding domain and C-terminal domain [1]. The microtubule binding region is composed of 3 to 4 Pro-Gly-Gly-Gly repeats, which help Tau protein to bind to the outer surface of microtubules, promote the assembly of microtubules, and participate in axon transport. The N-terminus contains a different number of inserted sequences, which protrude from the surface of the microtubules to contact other cytoskeleton components and cell membranes, and play an important role in maintaining the stability of axons. The difference in the number of repetitive sequences in the microtubule binding region and the difference in the number of N-terminal inserts have resulted in six different isoforms of Tau protein [2]. The distribution area of Tau protein is mainly concentrated in the neurons of the frontal lobe, temporal lobe, hippocampus and entorhinal area of the brain, as well as the axons of peripheral nerves.

Tau protein is a kind of microtubule-associated protein, which can be used as the core of early microtubule assembly after binding to tubulin, promoting other tubulins to extend and aggregate on this core to form microtubules, prevent depolymerization, and maintain the stability of its structure, maintaining the distance between microtubules, affecting the protein kinase attachment points of neuronal axons and playing an important role in neuronal plasticity [3]. The tau protein can also create conditions for the growth and extension of axons by maintaining the stability of the microtubule

structure. In the cell, tau protein helps the microtubule organization center to transport mitochondria, lysosomes and other organelles and extracellular secretory vesicles [4]. In addition, tau protein is also involved in the formation of oligodendrocyte myelination and plays an important role in promoting the maturation of oligodendrocytes. The tau protein can also participate in the intracellular signaling pathway by binding to the 14-3-3ζ protein, and indirectly regulating the distribution and function of a variety of proteins in the cell. Abnormal expression of tau protein or abnormal post-translational modification will cause it to lose its stabilizing effect on microtubules, cause the neuronal function to degenerate, and cause corresponding neurological diseases [5].

1.2. Post-translational modification of tau protein

The post-translational modification of tau protein is an important way to regulate the structure and function of tau protein. The common ones include phosphorylation, glycosylation, acetylation, truncation, and peptidyl-prolyl isomerization.

Phosphorylation of tau protein mainly occurs in serine (Ser) and threonine (Thr) residues. There are few phosphorylation sites of Tau protein in the normal mature brain, with an average of only 2 or 3, while the phosphorylation sites of Tau protein in the brain of AD patients are as high as more than 40. Among them, the phosphorylation of Thr231 and Ser262 directly affects the binding of tau protein to microtubules [6]. At the same time, the phosphorylation sites of Tau protein may also undergo mutual regulation and affect the function of Tau protein [7].

Tau protein glycosylation includes N-glycosylation and O-glycosylation. N-glycosylation mainly occurs on the asparagine (Asn) residues of the protein, which is the glycosylation mode of hyperphosphorylated Tau protein. O-glycosylation mainly occurs on the side chain hydroxyl of Ser or Thr, which is the glycosylated form of normal Tau protein [8]. There are a large number of N-glycosylated Tau proteins in neurofibrillary tangles. N-glycosylated Tau proteins can promote the production of cellular oxidative stress, which may be the inducement of Tau proteins to form neurofibrillary tangles [9].

García-Sierra et al. [5] found that the lysine (Lys) at position 280 of Tau protein has p300 and SIRT1-mediated acetylation and deacetylation modifications. Acetylation at this site promotes the phosphorylation of Tau protein itself. Under the same conditions, the acetylated Tau protein is more likely to aggregate to form filaments than the deacetylated Tau protein.

Natural Tau protein is the substrate of a series of proteases, and protein fragments produced by protease digestion or other methods may accelerate the accumulation of Tau protein and the progression of neurodegenerative diseases [10]. At present, nine restriction sites have been identified for Tau protein, and the truncation of these nine sites is mediated by different proteases. The fragments produced by truncation accumulate faster than the normal size of tau protein, resulting in obvious cytotoxicity [11]. Therefore, the truncation of Tau protein has a certain correlation with the development of AD.

2. hyperphosphorylation of tau protein and AD

Now the medical community generally agrees that the deposition of Aβ protein in the human brain is the central link in the pathological changes of AD [12]. Aβ is mainly produced by the APP protein via the β-secretase pathway. There may be a corresponding regulatory mechanism between the hyperphosphorylation of Tau protein and the production of Aβ. Studies have found that in patients with cognitive impairment whose cerebrospinal fluid hyperphosphorylated Tau protein (p-Tau) is negative, there is no correlation between the Aβ content of cerebrospinal fluid and cerebral cortical atrophy. However, patients with positive p-Tau will have a negative correlation between the degree of cerebral cortex atrophy and the expression of Aβ in the cerebrospinal fluid, suggesting that the existence of p-Tau is an essential factor for Aβ to affect cortical atrophy [13]. On the other hand, the abnormal secretion and accumulation of Aβ by nerve cells activates Tau protein kinase, promotes Tau protein phosphorylation, triggers the chronic inflammatory response, activates cell apoptosis, produces incompletely metabolized free radicals. This will cause an imbalance between the oxidation

and antioxidant effects in neuronal cells, which will lead to the death of a large number of neurons and glial cells.

Aβ needs to rely on Tau protein to produce downstream toxicity [14]. When Aβ acts on hippocampal neurons lacking endogenous Tau protein alone, it cannot cause degenerative changes in nerve cells. Another study showed that only reducing the level of Aβ in AD mice could not alleviate their learning and memory impairment, and only reducing Aβ and Tau protein at the same time could improve the cognitive function of AD mice. Aβ protein monomer can affect the transport of neuronal axons in the early stage of brain tissue lesions, inhibit the formation of ATP in mitochondria and the normal function of neurotrophic factor receptors in axons [15]. Under physiological conditions, only reducing the expression level of Tau protein cannot well repair the transport function of neuronal axons. In the presence of Aβ protein, the down-regulation of Tau protein expression showed obvious neuroprotective effects. It shows that reducing Tau protein can prevent the axon transport disorder mediated by abnormal expression of Aβ without affecting the axon transport function [16]. In addition, Tau protein is also involved in regulating the abnormal excitatory effects of Aβ and non-receptor tyrosine protein kinase Fyn on neural networks [17]. In addition, the hyperphosphorylated tau protein may also participate in the occurrence of AD by interacting with 14-3-3ζ protein [18], interacting with the multifunctional protein p62, and directly acting on it.

3. Visualization tools of Tau protein

3.1. Tau Super-Resolution Imaging

Ordinary optical microscopes are hard to distinguish objects with a distance of <200 nm, which makes it difficult to observe proteins and organelles in cells. However, the latest development in the field of microscopy, super-resolution microscopy (SRM), makes this possible. This technology has a spatial resolution of 20-50 nm [19], and has wide applications in different fields of life science research. One of the newest super-resolution techniques is Stimulated Emission Depletion Microscopy (STED). The main advantage of this method is that it can integrate the properties of fluorescence microscopy while avoiding the diffraction limit. The STED microscope was used to study the distribution of the physiological tau protein in the mossy fiber synapses in the CA3 hippocampus of the adult mouse brain [20]. Studies have shown that tau is unevenly distributed and rather sparsely localized at the level of the axon microtubules, about 200 nm away. This study shows that physiological tau does not saturate axonal microtubule (MT) binding. At the same time, STED can also visualize tau with a resolution of 77 nm in post-mortem AD sections of the human brain [21]. Immunofluorescence with the phosphorylated tau (p-tau) primary antibody 12E8 followed by STED imaging showed that the protein is mainly present in filaments, which are characterized by a smooth ribbon-like structure. The co-localization of tau with stable and dynamic microtubules and actin filaments was observed at the level of fixed growth cones rotation [22]. The use of small hairpin RNA (shRNA) to reduce the tau level does not affect the cone morphology, but leads to a reduction in axon outgrowth and growth cone rotation. Human excitatory cortical neurons from FTD patients were used to study the effect of autosomal dominant MAPT P301L and IVS10+ 16 tau mutations on protein function. In FTD-MAPT neurons, hyperphosphorylated tau is incorrectly localized in the cell body and dendrites; In addition, three-dimensional (3D) STED experiments show that an abnormal microtubule organization is the cause of the core deformation in FTD neurons [23].

The *Stochastic Optical Reconstruction Microscope* (STORM) was first introduced in 2006. It is an effective method of localizing proteins, achieving image resolutions below 20 nm. Using this technique, in the tau fibers labeled with ATTO-647 dye, the exogenous tau fibers collected on the neuron membrane were examined by imaging the hippocampal neurons. Although the density of exogenous tau fibers bound to neurons increases over time, the radius of the cluster is not affected. STORM was combined with neuropathological immunohistochemical methods to display p-tau in the prefrontal, parietal and temporal cortex of AD patients with a resolution of <50 nm [24].

Tau aggregates occur in filamentous structures at the axon level, while in somatic cells they have a honeycomb structure, most likely due to the presence of proteins and organelles. Finally, single-molecule localization microscopy (SMLM) was used to examine nanoscale tau tissue under physiological and pathological conditions [25].

From the various aspects of tau protein biology, the above content shows how super-resolution imaging methods can unravel the unknown side of tau structure and function under both physiological and pathological conditions. More recent SRM studies seem to prefer a non-uniform tau distribution along microtubules rather than a diffuse distribution [26]. One possible explanation for this is that previous research was mainly carried out by traditional optical microscopy methods, which could not resolve structures with distances <200 nm. SRM is uniquely useful for in situ monitoring of protein dynamics in fixed and living specimens. It can be used in the future to study the early stages of protein aggregation and its dynamics over time. However, some technical issues such as long-term imaging, fluorophore bleaching and sample thickness have yet to be overcome.

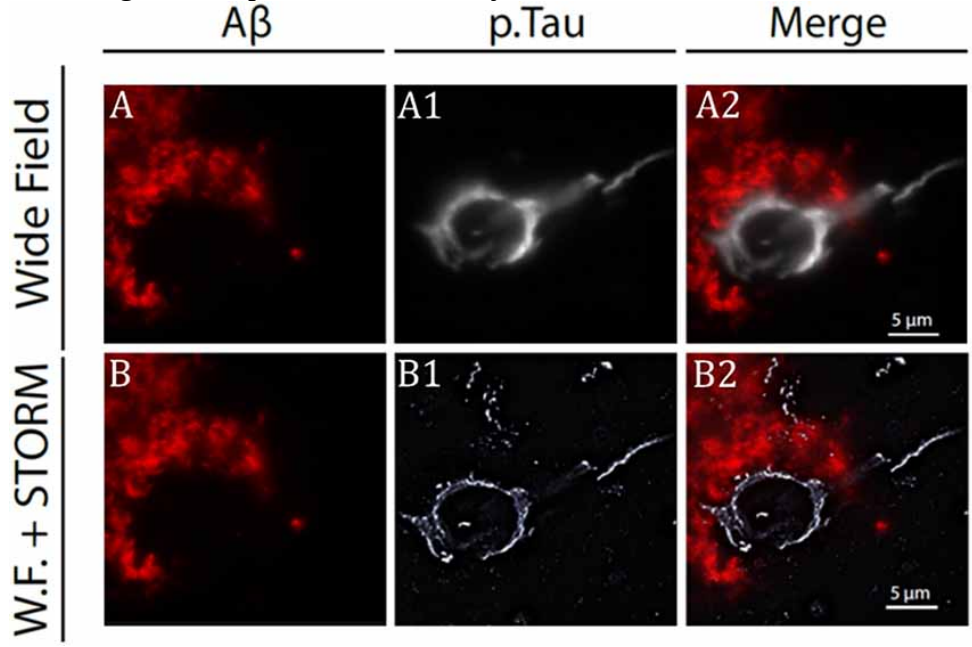

Figure 1. Stochastic optical reconstruction microscopy (STORM) image of beta-amyloid plaque and neurofibrillary tangles (NFT) in Alzheimer's disease (AD) patient brain slice. (A) Confocal microscopy image of beta-amyloid aggregates (in red) and neuro-fibrillary tangles (A1) from an AD patient brain specimen. Super-imposed images are shown in panel (A2). Same senile plaque as in panel (A), imaged by combining fluorescence microscopy for beta-amyloid aggregate (B) with STORM microscopy for NFT (B1). Super-imposed images are shown in panel (B2). Note the difference in resolution between image in panels (A2) and (B2) [26].

3.2. Near-Infrared Probes

Inspired by the above-mentioned work on curcumin analogs, Boris Schmidt's group synthesized many curcumin-like donor-acceptor-donor compounds (D-A-D) with core structures made of pyrazine, pyrimidine or pyridazine. Among them, the affinity of the three pyrimidine-containing compounds 1a-c to tau tangles is significantly higher than that of Aβ, and the affinity of 1a-c is 13.5, 9.17, and 26 times that of Thiazine Red R, respectively [27]. However, in the tissue section, 1a showed efficient staining of Aβ plaques without showing any fluorescence from NFT, while 1c showed no fluorescence. This observation is due to the lack of human tau protein in the specific AD mouse model used, in which the compound does not appear to have an affinity for mouse tau. The substitution of the dimethylaniline structure on the curcumin scaffold is the key compound in the development of the fluorophore, and the fluorescence intensity increases after incubation with the aggregated K18 protein, including the 4-repeat domain [28]. Among these compounds, compound 3 showed a 22.9-fold turn-on of fluorescence in solution, and stained aggregates of tau 2N4R-GFP

(green fluorescent protein) conjugates in vitro. However, this study did not conduct in vivo experiments or tissue staining experiments, nor did it conduct Aβ competition experiments. Finally, a more lipophilic and brighter fluorescent probe was constructed (5) by aromatizing the β-diketonate of curcumin structure. The curcumin-like D-A compound 5 combines the high opening rate of tau fibrils with the 14-fold selectivity of tau for Aβ and the emission maximum of 660 nm in NIR. This molecule can show excellent overlap with fluorescently labeled antibodies in human AD brain slices that show highly phosphorylated tau protein, while only showing low Aβ plaque staining in the core of the densest plaque. [29]

BODIPY dyes have been used as the preferred scaffold in fluorescent probes design because they are relatively easy to replace and usually have strong fluorescent properties [30]. Lin et al. proposed BD-tau. After adding tau protein aggregates to PBS, the fluorescence of this compound was enhanced up to 5.5 times, and the maximum emission wavelength was 590 nm. BD-tau shows good selectivity for tau pre-aggregates and bovine serum albumin, but a 3.5 to 4-fold increase in fluorescence was also observed in the presence of A or insulin fibrils. BD-tau did show significant co-localization in the brain slices of Tau-expressing cells and tau disease mouse models. Verwilst et al. described the synthesis of two probes (Tau1 and Tau2) based on BODIPY that are selective for tau [31]. The solution test in the presence of tau protein aggregates or Aβ fibrils showed that at 650 nm, the fluorescence enhancement near Tau 1 and Tau 2 were 6.4 and 9.3 times, respectively, while in the case of Aβ-fibrils almost no enhancement was observed. In in vitro experiments, Tau 1 proved that NFT is an effective marker in the acute and transgenic mouse models of tau disease and AD, and has the least core markers for dense core senile plaques. Finally, the authors proved Tau 1's ability to cross the blood-brain barrier and distinguish the state of tau aggregation in vivo in a transgenic mouse model.

K. Peter R. Nilsson's group investigated the effect of luminescent conjugated oligothiophene and proved that it can distinguish NFT and age spots on the spectrum [32]. Further work compared four thiophenes, five thiophenes, six thiophenes and seven thiophenes (q-FTAA, p-FTAA, hx-FTAA and h-FTAA, respectively) and confirmed the importance of terminal carboxylic acids [33]. Among all the probes, p-FTAA has a different solvent discoloration behavior from other probes, showing the highest dependence on solvent polarity and viscosity, and has a large Stokes shift (110nm). In another study, HS-68 showed great changes in spectral results when combined with tau tangles, and there was an age-dependent emission wavelength, with blue-shifted emission in NFTs of older mice and senile plaques. In addition, the oligothiophene-PBB3 hybrid bTVBT1-5 showed a maximum emission in the range of 600-630 nm, showed no double staining for NFT and senile plaques, but a very high selectivity for tau aggregates [34].

There is also a report of a strategy in which small tau antibody fragments (scFV) are coupled with near-infrared fluorescent probes [35]. Compared with full-length antibodies, scFv antibodies are smaller in size, offer the ability to target specific tau epitopes, and are more selective than β-sheet dyes. After the NIR-labeled scFv235 was injected into the two tau protein disease mouse models, the in vivo imaging system (IVIS) experiment showed strong and stable signals from the brains of JNPL3 and htau/PS1 mice, compared with the control animals. In addition, older mice of both genotypes showed higher signals compared with young mice, which further indicates the specificity of scFv 235 antibody [35].

3.3. Positron Emission Tomography (PET) Tracers

In order to conduct longitudinal studies and evaluate the effects of treatment at the aggregate levels, one must be able to repeatedly observe tau inclusions in the living human nervous system. [^{11}C] Pittsburgh compound B ([^{11}C]PIB) was used for the first time for PET imaging of brain contents characteristic of neurodegenerative diseases in human and which can detect β-amyloid deposits in living brains [36]. Several PET tracers were then developed for the polymerization of Tau, such as [^{11}C]PBB3, [^{18}F]AV-1451, [^{18}F]THK5351 and [^{18}F]PI-2620. Most tracers show a high affinity for the Tau content, but poor detection of β-amyloid deposits [37]. However, there are also some off-target effects. For example, non-specific retention of [^{11}C]PBB3 in the dural venous sinuses has been

observed [38]; the retention of [^{18}F]AV-1451 in the choroid plexus of control individuals also reflects the off-target binding [39]. The most worrying thing is that [^{18}F]AV-1451 and [^{18}F]THK5351 have been described in the semantic variation of primary progressive aphasia, a form of frontotemporal dementia that is not related to Tau inclusion bodies [40].

In the study, compared to the first generation tracers, the second-generation Tau PET tracers ([^{18}F]PM-PBB3, [^{18}F]MK-6240 and [^{18}F]Pl-2620) showed less off-target binding. Future autopsy studies need to determine the binding targets of these ligands. In addition, the combination of PET imaging and graph theory reinforces the belief that tau pathology can undergo proliferation across neurons [41]. Among them, PBB3 visualizes the Tau pathology of AD and non-AD Tauopathies [38]. In contrast to PBB3, earlier results showed that AV-1451 only binds to filaments from non-AD tauopathies and has a low affinity. According to reports, AV-1451 failed to visualize the Tau content in the human P301L Tau transgenic mouse strain [39]. However, it is possible to map Tau inclusions with [^{11}C]PBB3 in mouse models of Tau proteinopathy (PS19 and Tg4510 lines) [38]. It has been shown that both AV-1451 and PBB3 detect extracellular Tau content in the AD brain. We believe that the aggregated Tau in extracellular tangles closely matches to the structured filament cores.

4. Conclusion

Information such as tau structure, positioning and self-aggregation mechanisms, interaction with proteins and organelles depends on various imaging methods. These technologies are still in active development due to some limitations that need to be overcome. Fluorophore-conjugated antibodies or synthetic compounds have been used to label proteins to enhance these techniques. In the future, it is likely to improve the SRM labeling method combined with label-free microscopy [42] to overcome these problems. The work reviewed here shows that NIR-based probes have a fairly high specificity in vitro and in vivo. However, the low quantum yield and the solubility of the compound must be considered. Cryo-electron microscopy has also proved to be an effective tool for measuring the high-resolution structure of Tau filaments. Future work will focus on distinguishing the morphological differences of different amyloid filaments, which in turn will provide information on the mechanism of prion-like spread of protein aggregates. These new imaging methods with information about the structure of tau will make a huge contribution to clinical and basic tau research.

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