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NEURAL REGENERATION RESEARCH
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Lei Cao, Bin Ji, Ruiqing Ni*

Neurodegenerative diseases, including Alzheimer's disease (AD), frontotemporal dementia, Parkinson's disease, and dementia with Lewy bodies, represent tremendous unmet clinical needs. A common feature of these diseases is the aberrant cerebral accumulation of pathological protein aggregates, affecting selectively vulnerable circuits in a disease-specific pattern. Earlier studies have established a relationship between abnormal aggregation and neuronal dysfunction or loss, suggesting multifactorial pathogenesis mechanisms in these neurodegenerative disorders. Developing disease-modifying drugs requires a thorough molecular understanding of how the proteinopathies progressively spread and the link to neurodegeneration and cognitive impairment. Preventing and removing pathological protein aggregates has shown potential as an effective therapeutic strategy for these proteinopathies. Immunotherapies have demonstrated slowing the rate of cognitive decline by effectively removing pathological amyloid-beta (Aβ) deposits in patients with AD. Several immunotherapeutic approaches targeting tau, alpha-synuclein, and TAR DNA-binding protein 43 for the treatment of neurodegenerative disorders, including AD, primary tauopathies, alpha-synucleinopathies, and amyotrophic lateral sclerosis, are currently in clinical trials. Advances in neuroimaging technology have enabled the noninvasive detection of physiopathological events in the brains of living patients and disease animal models. Positron emission tomography (PET) and single-photon emission computed tomography (SPECT) with radioactive ligands for protein aggregates such as Aβ, tau, and alpha-synuclein with β-sheet structures have facilitated the early and differential detection of AD and primary tauopathies. Aβ and tau PET have demonstrated their clinical utility and validity and are considered surrogate efficacy endpoints in clinical trials of pharmacological or emerging nonpharmacological treatments. In the era of disease-modifying therapy, imaging biomarkers are expected to play an increasingly important role in early diagnosis, patient stratification, and monitoring, in addition to fluid biomarkers.

Animal models such as transgenic/knock-in/inoculated rodents recapitulating these features have been instrumental in deciphering the underlying disease mechanisms, although it is known that differences exist in the conformation of fibrillar aggregates between patients with disease and models. For *in vivo* imaging in animal models, a single-scale approach has often been used and provides limited information in the understanding of multifactorial diseases. This perspective highlights recent developments in the non-invasive imaging of proteinopathies in animal models.

Ligands: Antibody-based ligands: The ligands for proteinopathy imaging are mainly characterized as small molecules that detect the β-sheet structure, which are used in the clinical setting; and antibody-based ligands bind specifically to specific epitopes. Depending on the imaging modalities and imaging mechanism, the commonly used ligands have fluorescence emitting properties for fluorescence imaging, are suitable for photoacoustic imaging or magnetic resonance imaging or are labeled with radionuclei such as [^{11}C]/[^{18}F] for PET imaging. Antibody-based Aβ targeted ligands are sensitive and specific for detecting Aβ pathology in mouse models of AD but face challenges in blood-brain barrier entrance. To improve the brain uptake of Aβ and alpha-synuclein antibody fragments, transferrin receptor, which enhances receptor-mediated transcytosis, has been used as an effective approach for immunoPET/SPECT and application in AD and Parkinson's disease animal models (Sehlin et al., 2016). In addition, a recent study demonstrated *in vivo* fluorescence imaging using a single-domain antibody-based ligands of smaller size that do not require additional facilitators for crossing the blood-brain barrier (Jiang et al., 2023; **Figure 1**).

Near-infrared (NIR) II fluorescent amyloid imaging ligands: Most of the available amyloid fluorescence imaging ligands reside below the emission wavelength of 950 nm. The fluorescence signal is affected by intense light scattering and signal cross-talk with superficial tissues, rendering quantification of the distribution of ligands in deep brain areas of the animal model difficult. In addition, given that autofluorescence from endogenous molecules in the brain may hamper the accuracy, the practical efficacy of such a method needs to be taken into consideration. NIRII (1000–1350 nm) amyloid binding ligands have shown great potential in noninvasive imaging in living animals with greater penetration depth, signal-to-noise ratio, and sensitivity (Miao et al., 2022). Moreover, ligands with aggregation-induced emission properties are expected to not only support the *in vivo* detection of amyloid aggregate distribution in the brain with anti-quenching properties but also provide theranostic possibilities and even dual-targeted therapy, such as targeting reactive oxygen species and amyloid aggregated simultaneously.

Structure-based artificial intelligence (AI)-assisted screening: AI has revolutionized the field of medical imaging, influencing all processes from ligand discovery, screening, optimization of binding properties, pharmacodynamics and pharmacokinetics modeling, as well as streamlining image processing, analysis, and reporting (**Figure 2**). Rapidly advancing AI-assisted screening empowers the rapid development of imaging ligands based on the known structures of amyloids. Through the utilization of deep learning algorithms, AI can assist in identifying potential candidate molecules or antibody fragments with high binding affinities and specificity for pathological proteinopathies of specific conformations. This not only expedites the screening process and reduces the cost and time but also enhances the accuracy of the discovery step. Recent cryo-electron microscopy studies have revealed structural polymorphisms and disease-specific folding of amyloids in different diseases, such as alpha-synuclein in multiple system atrophy and Parkinson's disease (Yang et al., 2022). Cryo-electron microscopy enables us to shed light on the potential multiple binding sites for imaging ligands such as the tau ligand PM-PBB on tau fibrils from primary tauopathies and AD brains (Tagai et al., 2021) as well as the discovery of the alpha-synuclein ligand F0502B on alpha-synuclein fibrils (Xiang et al., 2023). Combining AI-assisted screening, optimization, and experimental assays, including binding property characterization and validation, will speed up the development cycle, particularly for challenging targets without clinically available ligands such as alpha-synuclein and TAR DNA-binding protein 43.

Organ-on-a-chip: Organ-on-a-chip technology has emerged as a transformative approach for studying complex physiological processes before *in vivo* imaging in animal models (**Figure 1**). It promotes the 3R principles (replace, reduce, refine) for the development and use of alternatives to animal experiments and has started to gain regulatory acceptance for toxicity testing. For imaging ligands targeting proteinopathies, the organ-on-a-chip platform allows for *in vitro* and *in vivo* extrapolation, i.e., to investigate the ligand uptake, distribution, and binding kinetics even in multiple organs in a physiologically relevant environment. This immensely reduces the need for *in vivo* imaging in animal models for toxicity as well as for physiologically based pharmacokinetic/pharmacodynamic studies.

Pushing the imaging resolution limits: In small animal models, PET/SPECT is able to contribute to new ligand development and provide a strong prediction for clinical results by using clinically available techniques at the preclinical phases (Tagai et al., 2021). While PET provides excellent accuracy in quantification, the small animal PET system has a limited spatial resolution relative to the mouse/rat brain, hindering accurate mapping of amyloid/tauopathy in different brain regions. The quantification of PET images is further affected by the partial volume effect for quantification. PET/SPECT, advanced optical imaging in animal models of neurodegenerative diseases, provides compensatory information for deepening the understanding of disease mechanisms. Recent long-term *in vivo* imaging using photoacoustic/optoacoustic tomography (Chen et al., 2022; Ni et al., 2022; Vagenknecht et al., 2022; Deán-Ben et al., 2023) has enabled high-resolution, real-time *in vivo* imaging beyond the 1-mm penetration depth typical of microscopy methods. We recently developed an Aβ imaging pipeline using a fluorescence volumetric optoacoustic tomography platform assisted with the Aβ fibril binding oxazine derivative AOI987 (Ni et al., 2022). AOI987 absorbs far-red light, unlike hemoglobin, but eliminates interference from blood vessels, a common problem with this type of microscopy in transgenic arcAb and APP/PS1 mouse models of AD amyloidosis.

Optical two-photon microscopy offers excellent spatial resolution and longitudinal observation for identical pathology or cells, enabling the assessment of inclusion turnover and the response of individual cells at different distances from pathological inclusions. However, two-photon imaging is afflicted by a small (submm) field of view and limited depth penetration. One recently developed approach using large field-of-view light microscopy has provided an opportunity to enlarge the FOV while maintaining high resolution (6–10 microns). Assisted by the luminescent conjugated oligothiophene dye HS-169, which binds Aβ and tau, light microscopy resolved individual plaques in APP/PS1 and arcAβ amyloidosis mice as well as tau deposits in P301L tau mice *in vivo* in the cortex. The imaging depth issues in proteinopathy imaging in animal models remain to be solved, since a large proportion of tauopathy and the majority of alpha-synucleinopathy are located in subcortical brain regions such as the hippocampus or striatum and substantia nigra. Qin et al. (2022) recently developed an adaptive optics three-photon microscope based on analog lock-in phase detection for focus sensing and shaping. This method measures and compensates for both aberrations and scattering induced by specimens with high accuracy. The authors demonstrated *in vivo* imaging of microglia and hippocampal neurons at synaptic resolution in the aged AD mouse brain through an open skull window. With the recently developed NIR-II two-photon amyloid detection ligands such as ZW800-1C (Hou et al., 2023), further *in vivo* applications in imaging Aβ and tau aggregates in deep brain regions in animal models are expected. This approach will enable an *in vivo* understanding of the accumulation of amyloid/tau and the phagocytosis of aggregates and neurons mediated by microglia in a living mouse model and provide insight into the link between amyloid/tau, neuroinflammation, and atrophy in the hippocampus.

https://doi.org/10.4103/1673-5374.392886
How to cite this article: *Cao L, Ji B, Ni R (2024) Advances in non-invasive imaging of proteinopathies in animal models of neurodegenerative diseases. Neural Regen Res 19(10):2115-2116.*

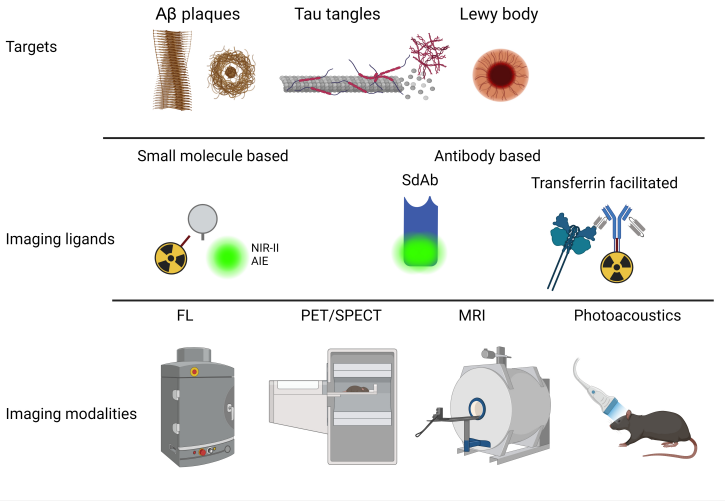

Figure 1 | **Non-invasive *in vivo* imaging of proteinopathies in animal models.**
Created with BioRender.com. Aβ: Amyloid-beta; AIE: aggregation-induced emission; FL: fluorescence; MRI: magnetic resonance imaging; NIR: near-infrared; PET/SPECT: positron emission tomography/single-photon emission computed tomography; SdAb: single-domain antibody; TfR: transferrin receptor.

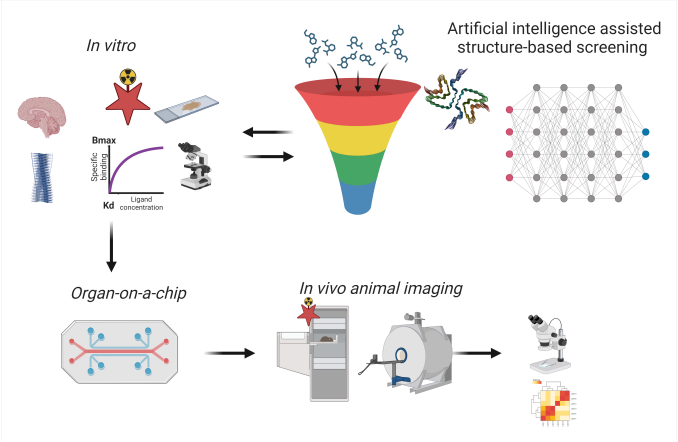

Figure 2 | **Scheme of the development of imaging biomarkers for proteinopathies in neurodegenerative diseases.**
Created with BioRender.com.

Contrast agent-free imaging: The aforementioned various imaging approaches are made possible with the assistance of imaging ligands. Contrast agent-free imaging of amyloid deposits has been feasible in recent years. Pansieri et al. (2019) developed approaches for *in vivo* and *in vitro* ultraviolet imaging of Aβ in a mouse model based on the ultraviolet-visible-near-infrared optical properties of amyloid fibrils. Using continuous-wave NIR fluorescence-enhanced diffuse optical tomography, the author demonstrated direct detection of amyloid deposits in the brains of living APP/PS1 mice at 18 months of age with abundant Aβ plaque accumulation. An increased *in vivo* NIR signal (excitation wavelength 690 nm) was measured using fluorescence-enhanced diffuse optical tomography and could also be seen using noninvasive 2D NIR imaging (Fluobeam 800) performed on the head of living mice. The ultraviolet-visible-near-infrared optical properties of amyloids open new research avenues for amyloidosis as well as for next-generation biophotonic devices. Whether a similar approach can be applied to image other amyloids, such as tau and alpha-synuclein, remains to be demonstrated.

In conclusion, there have been rapid advances in the noninvasive imaging of proteinopathies in terms of new ligands, AI-assisted discovery, and the development of new multiscale imaging setups with improved imaging resolution and depth. Therefore, it would help to understand the underlying disease mechanism and aid in the preclinical drug discovery.

No conflicts of interest exist between Changes Technology Corporation Ltd. and publication of this article.

RN received funding from Swiss Centre for Applied Human Toxicology, and Helmut Hortun Stiftung.

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Date of submission: August 12, 2023
Date of decision: November 7, 2023
Date of acceptance: December 1, 2023
Date of web publication: January 8, 2024

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C-Editors: Zhao M, Liu WJ, Qiu Y; T-Editor: Jia Y