Molecular Targets in the Rational Design of AD Specific PET Tracers: Tau or Amyloid Aggregates?

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Abstract: A major limitation in finding therapeutic solutions for Alzheimer’s disease (AD) has been the lack of a reliable method for early diagnosis of this devastating disease. Besides the development of biomarkers in biological fluids of patients, the search for a pathology-specific neuroimaging tools is critical at the present stage in which almost 30 million people suffer this disease worldwide. Several interesting approaches have been developed, however their clinical impact has been low. One of the difficulties has been to find the proper molecular tracers to specifically tag pathognomonic lesions in AD brain, including not only amyloid aggregates but also filaments of the modified microtubule-associated protein tau. In this review, we analyze the evidence towards developing pathology-specific diagnostic tools for AD. We analyze the current evidence and clinical implications of new imaging technologies for AD, and how tau hypothesis and the amyloid cascade hypothesis will impact on these scientific efforts in the near future.

Keywords: Alzheimer’s disease, PET radiotracers, brain neuroimaging, benzimidazoles, tau protein, amyloid beta.

INTRODUCTION

Over the last thirty decades, Alzheimer’s disease (AD) has become an increasingly difficult challenge, not only for the scientific community, but also for the healthcare systems and for AD patient’s families, as the number of patients increases and the frustration due to lack of effective treatments grows [1, 2]. Though there are several FDA-approved drugs for AD treatment, such as memantine and Acetylcholinesterase inhibitors (AChEIs), they are neither AD-specific nor etiologic treatments [1]. Furthermore, the current drugs for AD have shown debatable clinical efficacy [1, 3] especially in late stages of the disease. Thus, in spite of the scientific efforts of the last fifty years, AD has become an area of significant unmet medical needs. The failure in finding effective etiologic therapies for AD partially lies on the lack of pathology-specific diagnostic technologies. Today, there are numerous pharmaceutical companies developing novel treatments for AD, as it seems a promising medical market for the future. However, not much of these efforts are directed solve the bottleneck of finding accurate AD-specific diagnostic technologies [4]. Such technologies would allow medical researchers discriminating AD from other types of dementia and searching for of etiologic therapies more efficiently. AD and multi-infarct dementia are the two most common causes of dementia in the middle-aged and elderly people around the world. It has been estimated that only AD accounts for up to 75% of all dementia cases in U.S.A. and Europe. In the U.S.A. about five millions people are affected by this disease, and mortality is near 100,000 per year [1, 5]. AD constitutes nowadays one of the major health problems in the world (projections for year 2010 indicate that over 30 million people will have AD). In USA, the total cost of AD to the economy is over USD 174 billion per year [6, 7]. Thus, it is highly valuable to make scientific efforts in translating the current neuro-biological knowledge into reliable and non-invasive diagnosis tools. Nowadays, the diagnosis of AD is made according with the NIH-ADAD criteria [8] by the application of several neuropsychological tests that exclude other potential causes of dementia with a degree of accuracy ranging from 50 to 90% [9]. Thus, clinical diagnosis of AD can only be confirmed histopathologically, by the observation of significant amount of neurofibrillary tangles (NFTs) and senile plaques (SPs) in neo-cortex and hippocampus [9, 10]. NFTs, originally visualized in 1907 by Alois Alzheimer [1, 11], provided a pivotal impetus for the study of their molecular substrate. Paired helical filaments” (PHFs), formed by hyperphosphorylated forms of the tau protein, are the major components of NFT. The aggregation mechanisms of tau into NFT are extensively reviewed by other authors in this special issue [12, 13]. The other histopathological hallmark of AD are SPs, mainly composed of amyloid peptide, originated the most popular hypothesis on the etiopathogenesis of AD, the so called “Amyloid Cascade Hypothesis” [11, 14]. According to this hypothesis, the generation and self aggregation of Aβ peptide would be the primary pathological event leading to neurofibrillary degeneration and dementia in AD patients [14, 15]. Consistent with
these hypothesis, crossing FTDP-17 tau mutation P301L expressing transgenic mice with APPThg2576 (APP Swedish plus London mutations) mice, was found to exacerbate neurofibrillary pathology [16]. Accordingly, different groups including ours have published evidence linking tau pathology and the amyloid hypothesis. Specifically, we reported the activation of cdk5 and JNK-p38 systems in APPThg2576 mice [17, 18]. These and other findings related to SPs and NFTs have led to develop therapeutic and diagnostic approaches based on amyloid [19-24] and tau pathology [23-27]. In this review we analyzed the evidence towards developing pathology-specific imaging technologies for AD and how tau hypothesis and Amyloid Cascade Hypothesis will impact these scientific efforts in the near future.

RELEVANCE OF AMYLOID PATHOLOGY AND NFTS IN CREATING NEW IMAGING TECHNOLOGIES

As mentioned above, SPs are a hallmark of AD and thus have become a major target AD-specific imaging radiotracers. According to the Amyloid Cascade Hypothesis, SPs are extracellular deposits of insoluble amyloid fibrils, which are polymers of the amyloid-beta protein [11]. One characteristic form of amyloid plaque is often referred to as the “neuritic plaque” namely, an extracellular core of amyloid fibrils intimately surrounded by dystrophic dendrites and axons — some of which also contain PHFs — as well as by activated microglia and reactive astrocytes [11, 28]. Interestingly, according predictions derived from the amyloid hypothesis postulated by Hardy and Selkoe in the early nineties [11, 29], tangle formation would be a closer biological event to neuronal cell death compared to SPs formation [28]. This prediction, being confirmed in 1998 with the identification of tau mutations in FTDP17-T [30], raised the fact that NFTs could be an excellent target for AD-diagnostic imaging technologies. In spite of this fact, and probably because SPs are highly abundant in the brain of AD patients, a great body of work was done in the last twenty years, pointing out SPs as the main target for AD radiotracers [19, 21, 31]. However, clinical-to-pathological correlations have consistently demonstrated that the number of NFTs in the hippocampus and entorhinal cortex, and not the SPs plaques correlates best with the presence and the degree of dementia in AD [32-34] (reviewed by Ikbal [35]. In essence, the current evidence from molecular and clinical works suggests that tau pathology correlates well with cognitive decline.

At the same time, several amyloid-derived peripheral markers have been proposed, such Aβ oligomers levels in serum, blood cells [25, 26], and in CSF [21, 27]. These studies seemed promising towards new and successful diagnostic technologies. However, none of them have yet become a routine diagnostic tool. Thus, apparently many of the efforts towards therapeutic [22, 29, 34] and diagnostic [36] interventions based on the amyloid cascade hypothesis have either failed in becoming gold standard treatment/diagnostic tools, or are still waiting for more evidence [34, 37]. A classical example of this are the clinical data from the amyloid immunization, which taught us that a massive reduction of amyloid burden is not a miracle cure for AD, especially at late stages of the disease [1, 3, 34]. In the diagnostic area, the data from clinical studies apparently have not provided yet enough support to the amyloid cascade hypothesis [33, 37, 38]. In the current scenario, where imaging AD pathology has emerged as the right pathway to its early diagnosis, it is perhaps misleading to design experimental strategies to probe correlations between amyloid deposition and cognitive function. In this sense, Sperling and coworkers [38] have recently demonstrated that asymptomatic older individuals show PIB-positive areas and aberrant default network functional magnetic resonance imaging (fMRI) activity. In a different study by Jack et al., no correlation was found between amyloid deposition and cognitive decline [37]. In this context, several publications have demonstrated that cerebral amyloid aggregates, may not continue to accumulate during AD progress, and thus the amount of SP observed at any time point (including the autopsy) may reflect a competing processes of deposition and resolution of amyloid aggregates. These finding could explain why the PIB-compound and other similar PS-selective technologies are not been widely accepted in the clinical setting as radiotracers for AD, even after almost seven years of its pioneer clinical trial [21]. According to a recent clinical study on PIB imaging [36], AD diagnosis would be "...possible" but not "probable" and certainly not "definite..." if it is based on SP-selective radiotracers, such as PIB compound and others Fig. (1). Along this same line, several authors have reported that tracers tagging SPs can also efficiently tag cerebral amyloid angiopathy, which raises the fact that amyloid-selective tracers could reflect Aβ in cerebral vessels more than Aβ in brain areas involved in cognitive functions. All together, according to the current evidence in this field, it seems unlikely that amyloid-based imaging technologies will soon overcome the current limitations in the diagnosis of Alzheimer’s disease.

Fig. (1). Thioflavines and senile plaques selective benzothiazole derivatives. ThS, proposed structures for Thiflavine s; ThT, Thiflavine T; PIB, Pittsburg compound.
According with the neuroimmunomodulation theory on AD [2, 39, 40], a chronic-asymptomatic inflammatory process of the CNS is responsible for the earliest changes that precedes AD clinical onset in the vast majority of sporadic AD cases, including the formation of tau oligomers in the transenthorinal cortex. In this neuroinflammatory process, abnormal phosphorylation of tau and long-term activation of the innate immune system occurs, leading to cytoskeletal alterations such as tau protein aggregation and PHFs formation. Several factors contribute to trigger innate immune system alarm mechanisms resulting in the overproduction of cytokines such as tumor necrosis factor alpha (TNF\(\alpha\)) and others [23, 40-43] associated with progressive cognitive decline. In this context, we suggest that innovative diagnostic approaches for AD must be based, not only on the determination of hyperphosphorylated tau, or redox iron levels [23] but also on correlations with the ApoE4 allele [25], and more importantly, on the visualization of early pre-tangles of tau, as a more accurate way to assess AD pathological process. Studies from different authors, including our group [20, 44] have reported benzimidazolic and benzothiazolic compounds with voluminous moities in position 2 of benzimidazolic ring Fig. (2) display high affinity for pathological tau aggregates [20, 44]. This approach would allow clinical researchers to obtain maps of these brain lesions that are the major trademarks of this disease [22, 45].

COMBINED NTFS-MAPPING AND MICROARCHITECTURE-MRI: AN IDEAL TOOL FOR AD DIAGNOSIS

Magnetic resonance diffusion tensor-based imaging (DTI), also called “Tractography”, constitutes one of the newest and most promising \textit{in vivo} quantitative MRI techniques aimed to map white matter brain tracts. DTI has been successfully applied to several neuropsychiatric disorders [46, 47]. Tractography detects microscopic changes in water distribution within brain microstructure and is a highly sensitive -and specific- indicator of fiber tract integrity, organization and density of fiber bundles in brain white matter [47]. Therefore, DTI becomes an ideal non-invasive tool to be combined with early NFTs mapping, in order to detect AD-specific alterations of intra and inter hippocampal connections. This is specially relevant in the context of neuroimmunomodulatory hypothesis of AD [39, 48], according to which asymptomatic neuroinflammation and neuronal loss occur in early stages of AD. Thus, early changes in the brain microenvironment of AD patients should cause DTI-detectable abnormalities in the white matter. DTI abnormalities in several fiber tracts, such as posterior cingulated tracts and uncinated fasciculum, may reflect progression of AD-related to histopathological changes [49]. Fractional Anisotropy (FA), a common measure of white matter abnormalities, in the cingulum tract and hippocampus has been reported to correlate with the Mini Mental State examination (MMSE) score [50], arising a new promising avenue to measure cognitive deficits progression [49]. Also, a recent study combining DTI and Volumetry found AD specific alterations in the subventricular zone (SVZ), but not in other periventricular regions. This is relevant, since SVZ is one of the most important zones where proliferative stem cells are located in the adult brain. This study supports the AD hypothesis of an imbalance in the neurogenesis/neurodegeneration ratio, recently proposed by different authors including our group [39, 48, 51]. Another promising application for combined DTI-NFTs mapping is the possibility of improving differential diagnosis of dementias based on the evaluation of specific white matter alterations and tau pathology progression. In this sense, DTI has been suggested as a potential tool to describe different patterns of fiber integrity in the splenium of corpus callosum between AD and mild cognitive impairment patients [52]. Also, another potential application for combined DTI-NFTs mapping is to accurately differentiate Vascular dementia (VD) from AD cases based on the tracking regional changes in transcallosal prefrontal tract [53]. Taken together, these results suggest that combined DTI-NFTs mapping could become a powerful neuroimaging technique, not only for the early diagnose of AD, but also for correlating early stages of tau pathology with asymptomatic neuronal loss and early neuroinflammatory processes.

CURRENT TECHNOLOGIES FOR HIGH-THROUGHPUT EVALUATION OF NEW BRAIN IMAGING AGENTS

Biosensor Technologies

Surface plasmon resonance (SPR) based technologies have allowed rapid, label-free characterization of protein-
protein and protein-small molecule interactions (54). During the last decade, biosensors have become the gold standard method in industrial and academic settings, in which a pair of soluble binding partners is characterized in detail or a library of molecules is screened for binding against a protein. Classical and combinatorial methods of chemical synthesis will soon generate libraries of hundreds new potential AD imaging agents, which implies the need for efficient high throughput screening methods. However, it is not always possible to obtained radio-labeled versions of candidate compounds, which limits the efficiency of the screening for new imaging agents. As PHFs can be efficiently isolated from AD patients by immunological or chemical methods [55, 56] and immobilized on CM5 sensor chips [44], SPR represents a promising approach to screen new potential radiotracers for AD. Some important advantages of SPR approaches over the classical ligand-receptor assays are: (i) the high quality data in real time, (ii) no labeling requirements, (iii) detection of phenomena missed by radioligand assays [54] (Table 1). Recently, our group has designed an SPR-based method to compare the affinity of non-labeled benzimidazoles and thioflavine for PHFs and amyloid aggregates [44]. Protein density (PHFs or amyloid polymers) immobilized on a biosensor surface can be easily assessed by resonance units (RU). One RU represents the binding of 1 pg of protein/mm². This makes possible to compare relative affinities of non-labeled ligands for PHFs and amyloid aggregates [44]. Specific antibodies against PHFs and amyloid aggregates, thioflavine-S and congo red can be used to assess the integrity of immobilized tau and amyloid aggregates [44].

**Classical Radio-Ligand Assays**

Inhibition and saturation studies are routinely performed to measure the relative affinity and other kinetic parameters of new imaging agents. Brain-isolated PHFs, amyloid polymers [16, 17, 46], gray and white matter [46] are the most common targets used for ligand screening in these assays [19, 20, 31]. Inhibitions studies are normally used to determine the relative affinity of non-labeled compound respect to a radio-labeled one. Even though this by far the most widespread technology in ligand screening programs, it has important limitations that become more relevant when searching for selective PHFs imaging tracers. First, these conventional methods require having at least one radio-labeled compound with known (and high) affinity for the selected molecular target. Second, the dynamic ligand-protein equilibrium is totally disrupted during filtrations and temperature-manipulation steps, often done in these protocols. Third, inhibition assays assume that the non-labeled compounds share the same binding site(s) with the radio-labeled compound, which is likely, but not always true. This last issue is of great relevance in the case of brain-isolated PHFs, as these tau aggregates are highly heterogeneous entities. PHFs present in AD patients, are complex structures, not only composed of hyperphosphorylated/truncated tau protein [47], but also of ubiquitinylated and glycated isoforms of tau, mainly 3R and 4R variants [45, 48]. Thus, there is a high probability for the existence of many structurally different binding sites for imaging agents. Therefore, the screening programs based on inhibition studies, could easily underestimate the binding affinity of good candidates with different binding sites.

**Fluorescent Staining of Brain Tissue Sections**

This is probably one of the most handful technology in the search of new ligand for PET imaging, as it allows answering several questions in one experiment. The binding pattern of fluorescent molecules in brain parenchyma can be compared with those of specific antibodies against hyperphosphorilated and truncated forms of tau [47]. Thioflavine-T and Congo red are the most common dyes in

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<th>Technique</th>
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<tr>
<td><strong>Biosensor Technologies (Surface Plasmon Resonance)</strong></td>
<td>No radioactive or fluorescent labeling is required. It detects molecular phenomena missed by classical radioligand assays. It allows assessing direct interactions between ligands and protein aggregates. It can provide comparative kinetic data on amyloid polymers and PHFs fixed in the same chip.</td>
<td>It provides data on the ligands affinity but not specificity. Requires expensive technology and highly trained personnel.</td>
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<td><strong>Radioligand assays</strong></td>
<td>Relatively simple to set up.</td>
<td>It requires radiolabeled compounds and special facilities; kinetic data obtained varies depending on the experimental set up. Production of radioactive waste. High throughput not possible unless non-labeled molecules share same binding sites of radioactive compound (indirect interaction).</td>
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<td><strong>Autoradiography</strong></td>
<td>Provides data on specificity of the binding in the context brain parenchyma.</td>
<td>Radio labeled compounds and special facilities. Production of radioactive waste. Requires human brain sections, which are not always easily available.</td>
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<tr>
<td><strong>Fluorescent staining</strong></td>
<td>Provides data on affinity and specificity in the context of brain parenchyma. Requires fluorescent or fluorescently labeled compounds.</td>
<td>It requires fluorescent compounds or derivatization of the original molecule into a florescent derivative.</td>
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T and Congo red are the most common dyes in pathological staining of SPs and NFTs Fig. (3). However, PET tracer candidates are not always fluorescent or can not be fluorescently labeled, which limits its use only to fluorescent compounds.

CONCLUSIONS

The impressive increase in AD prevalence, along with the limitations in the therapeutic avenues available today, strongly indicate the need to develop reliable and safe biomarkers in biological fluids together with innovative pathology-specific neuroimaging technologies. For this later goal, it is critical to identify rational targets. Since most of the neuroimaging approaches directed to solely tag senile plaques already failed, due to the lack of correlation between amyloid plaques and the degree of cognitive impairment in AD patients -among other problems- it is worth pointing now research efforts to indentify pathological tau filaments as potential targets for early diagnosis of AD. PIB-compound developed recently have not been successful, perhaps because there is clear demonstration that asymptomatic older individuals show PIB-positive areas and aberrant default network functional magnetic resonance imaging (fMRI) activity, showing there is a lack of labeling specificity. Clearly, no correlation has been yet demonstrated between amyloid deposition and cognitive decline. Accordingly, a recent clinical evaluations on PIB imaging suggests that AD diagnosis would be not "probable" and certainly not "definitive..." if it is based on SP-selective radiotracers, such as PIB compound [36]. PIB compound has undoubtedly contributed to increase our knowledge on how AD-specific imaging technologies will impact the neurodegenerative diseases clinical settings. PIB-based technologies have also helped us to answer a seemingly straightforward and rather question: Is the amyloid burden correlated with cognitive decline in AD patients? This question has confronted “Baptists” and “Tautists” over decades and will probably remain as a continuous debate in this field of medical knowledge. In this context, we suggest that innovative diagnostic approaches for AD must be based, not only on the determination of levels amyloid peptides or its oligomers, hyperphosphorylated tau [24], or redox iron levels, but also on correlations with the ApoE4 allele, and

Fig. (3). Histopathological staining of brain sections from AD patients: (A-B) Multiple NFTs in entorinal cortex stained with PHF-1 antibody. (B) Magnification of the central square in panel A, showing the excentric neuron nuclei surrounded by a NTF (red arrow) and abundant neutritic lesions (violet arrows) at 40x magnification power. (C-D) Multiple plaques stained with 6E109 antibody. (D) shows a magnification of the rectangle in panel C. (E) brain section of AD cases stained with lansoprazole. (F) Brain section adjacent to section of panel E, panel F shows tangles and senile plaques stained with Thioflavine S.
more importantly, on the visualization of early pre-tangles of tau, as a more accurate way to assess AD pathological process. Finally, other cutting edge technologies, such as magnetic resonance diffusion tensor-based imaging (DTI), constitutes one of the most promising in vivo quantitative MRI techniques aimed to map white matter brain tracts. Thus, based on the current molecular and clinical evidence, we suggest that combined NFTs/DTI mapping could become especially relevant for early AD diagnosis.

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