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RESEARCH ARTICLE

Targeting Calpain-2 for Alzheimer's Disease Treatment

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ABSTRACT

There is an urgent need for treatments for sporadic Alzheimer's Disease (sAD). Although antibodies removing ß-amyloid have recently been shown to slow disease progression, the degenerative course continues. Thus, there is a need for strategies that intervene early in the degenerative process, before irreversible damage is done to neurons (e.g., by autophagic degeneration). This review will summarize the evidence indicating that targeting calpain-2 with a selective inhibitor might represent a novel strategy for the treatment of sAD. Calpains are neutral proteases that are activated by intracellular calcium. The two main isoforms are calpain-1, which is activated by low, micromolar levels of calcium and generally has beneficial effects for cellular health, and calpain-2, which is activated by high, almost millimolar levels of calcium and mediates many of calcium's toxic actions. Calcium signaling becomes dysregulated with advancing age due to loss of regulatory proteins such as calbindin, and is pronounced in sAD brain tissue, including signs of calcium leakage from the smooth endoplasmic reticulum (SER) through phosphorylated ryanodine receptors (pRyR2). Both calpain-1 and calpain-2 are elevated in AD brains and herald the rise in tau pathology, but only calpain-2 is localized with neurofibrillary tangles (NFTs) and pretangles. Calpain drives a spectrum of AD-related pathologies, and in particular, calpain drives tau hyperphosphorylation by cleaving, and thus disinhibiting, kinases central to tau hyperphosphorylation, i.e., GSK3β and cdk5, as well as increasing Aβ formation and autophagic degeneration. Thus calpain-2 inhibitors may reduce a spectrum of sAD pathology, protecting neurons at very early stages of disease.

1. Introduction

Current treatment strategies for Alzheimer's disease (AD) have focused on targeting amyloid or tau pathology, the traditional hallmarks of AD neuropathology. Although this approach has begun to have some success in slowing progression, it has not cured or halted the disease. Ideally, we need to understand what causes the neurodegeneration and accompanying tau and amyloid pathologies, in order to intervene very early to prevent the initiation of the pathological processes, especially since even early stage tau and amyloid pathology can have toxic effects in neurons. However, very early intervention would require a very benign treatment that potentially could be taken for decades by healthy individuals, without interfering with normal cellular functioning. The current review describes the importance of calcium dysregulation as an early etiological factor in the development of AD, and the potential use of selective calpain-2 inhibitors to safely reduce pathological mechanisms prior to the onset of irreversible neuronal damage.

Calpains are soluble, neutral, calcium-dependent proteases, i.e., they cleave other proteins, and they are activated by intracellular calcium in the cytosol. While there are 15 members of the calpain family, two ubiquitous isoforms of calpains are calpain-1, which is activated by micromolar concentrations of calcium that occur under normal physiological conditions, and calpain-2, which is activated by almost millimolar concentrations of calcium. In addition, recent studies have shown that calpain-1 activation is neuroprotective while calpain-2 mediates many of calcium's toxic actions[1]. As reviewed below, dysregulation of calcium with advancing age and/or inflammation leads to noxious levels of cytosolic calcium and activation of calpain-2, which in turn drives tau and amyloid pathology, synapse loss, and autophagic neurodegeneration. We propose that selective inhibition of brain calpain-2 may reduce early pathological events while leaving beneficial calcium actions intact, thus protecting neurons from future insults.

2. Alzheimer's disease pathology, and the amyloid vs. tau hypotheses

AD is a progressive neurodegenerative disorder characterized by memory loss and cognitive deficits, which ultimately lead to severe dementia[2]. AD is the most prominent form of dementia in aged people, and the number of Americans suffering from the disease is projected to reach about 15 million over the next few decades[3,4]. AD brain pathology is characterized by the presence of senile plaques consisting of aggregated extracellular β-amyloid peptide, and intracellular neurofibrillary tangles, which are composed of hyperphosphorylated tau proteins[2]. In addition, AD is associated with synapse loss and neuronal death in hippocampus, entorhinal cortex, basal forebrain, and neocortical association cortices[5]. Since AD affects the health and lives of so many people, there is a clear urgency to understand the molecular/cellular processes underlying the disease and to develop treatments that can slow down its progression and ultimately prevent or reverse it.

Although the brain pathology associated with AD is now widely agreed upon, the underlying mechanisms remain unclear and debated. For years, the similarities between the pathology in familial, early onset, autosomal dominant forms of AD, and the sporadic forms of AD, have suggested the existence of a common etiology. Familial forms of AD appear to be due to mutations in genes encoding the amyloid precursor protein[6], and presenilin-1 and presenilin-2[7], which all result in increased levels of β-amyloid peptides in brain[8]. However, familial forms of AD account for less than 5% of all AD cases[9]; thus, it is clear that the vast majority of AD patients develops AD sporadically (sAD).

The genetics of familial forms of AD have led to the amyloid hypothesis of AD, which postulates that Aß accumulation initiates the pathological process and drives tau pathology, which results in synaptic and dendritic dysfunction, and ultimately neuronal death. This hypothesis has led to the use of numerous mouse models with mutations in various amyloid-related genes to reproduce the human pathology with some degree of success[8]. This has also inspired the development of numerous, potential therapies targeting amyloid deposition or clearing. However, most of these have failed in clinical trials[10-12]. Only recently, lecanemab, an antibody against Aß, which removes amyloid from AD brains, has shown a small but highly significant slowing in cognitive decline[13], supporting the amyloid hypothesis. However, other compounds, successful in removing brain amyloid pathology, have had limited or no success [14-16], and lecanemab does not stop progression of the disease and does not appear to help patients with aggressive forms of disease, e.g., those who are apoE4 homozygotes[13].

Although much of the field has focused on the amyloid hypothesis, extensive postmortem evaluations of sporadic AD brains by Braak and colleagues have repeatedly shown that tau pathology is initiated 10 years *before* the appearance of amyloid plaques[17]. Moreover, the extent of tau pathology, and not amyloid pathology, correlates positively with the degree of loss of grey matter and of cognitive impairment[18]. The time-course and regional distribution of tau pathology are also different than those of the Aß accumulation and are consistent with the progression of cognitive symptoms. Tau pathology is particularly pronounced in glutamatergic projection neurons with extensive cortical-cortical connections and not in GABAergic interneurons. In cortex, tau pathology is initiated in layer II of the entorhinal and perirhinal cortices, and then proceeds to interconnected limbic and association cortices, as well as hippocampus. This progression likely involves the trafficking of phosphorylated tau between connected neurons[19], which are in a vulnerable state, e.g., with calcium dysregulation, as described below.

Recent data suggest that tau pathology may initiate or hasten amyloid pathology. Immunoelectron microscopy has shown that tau hyperphosphorylation and aggregation on microtubules could drive Aß generation in neurons due to an "endosomal traffic jam', as it traps the amyloid protein precursor (APP) in endosomes containing ß-secretase, increasing its cleavage to Aß[20]. As Aß in turn drives tau phosphorylation, this would establish a vicious cycle of neuropathology[21].

3. Calcium dysregulation as an early pathological event in AD

AD specifically afflicts the limbic and association cortices, and aging is the largest risk factor for the disease, with inflammatory conditions (e.g., insulin insensitivity, traumatic brain injury) contributing additional risk. Why are glutamatergic neurons in the limbic and association especially vulnerable with advanced age and inflammation? Studies from a number of disciplines indicate that these neurons express the molecular machinery to magnify calcium signaling needed to sustaining neural representations in memory stores[22,23]. However, dysregulation of calcium with advancing age and/or inflammation renders these neurons particularly vulnerable to AD pathology. The calcium hypothesis of AD has been discussed for decades[24-27], with early studies of AD brains by Nixon and Mattson showing that dysregulated calcium is likely a major initiating factor in AD pathology[28,29], and recent research from primate models has reinforced their groundbreaking work.

The pyramidal cells in layer III of the dlPFC have been an important focus of research, as these neurons are critical for higher cognitive functions[30,31], and are selectively vulnerable to tau pathology and neurodegeneration in AD[32,33], thus providing important clues about AD etiology. Studies of these neurons in rhesus monkeys have shown that they express the molecular machinery to magnify calcium signaling in dendritic spines at glutamate synapses (Fig. 1), where cAMP-PKA signaling increases calcium release from the smooth endoplasmic reticulum (SER), which in turn leads to more cAMP formation, thus producing a feedforward signaling[31]. Rhesus monkeys are all apoE4 homozygotes[34], a major risk factor for AD, which make them a particularly interesting animal model to study the etiology of AD. In addition to internal calcium release, cAMP-PKA signaling also magnifies calcium entry through NMDAR[35], and voltage-gated calcium channels[36], which can in turn further increase calcium-mediated calcium release from the SER[36]. Interestingly, apoE4 also increases calcium entry at these sites[37-40], suggesting that this may be one of the important ways apoE4 increases risk of AD. In the healthy, young dlPFC neurons, cAMP magnification of calcium signaling is tightly regulated by phosphodiesterases (PDE4s), which degrade cAMP, and by calbindin, which binds cytosolic calcium[41]. However, with advancing age and/or inflammation, PDE4s and calbindin are lost from layer III dlPFC dendrites, leading to excessive cAMP-PKA-calcium signaling. This process is worsened by PKA phosphorylation of ryanodine receptors (pRyR2), which flux calcium out of the SER, leading to calcium leak from the SER into the cytosol[42]. Studies of aging monkeys show that calcium leak through pRyR2 highly correlates with the initiation of early stage tau phosphorylation, e.g., by PKA at serine 214 (Fig. 1)[43]. Tau phosphorylation at S214 causes tau to change shape and detach from microtubules, and primes it for hyperphosphorylation by other kinases, especially GSK3ß and cdk5. As described below, these events are particularly damaging when cytosolic calcium levels are sufficient to activate calpain-2.

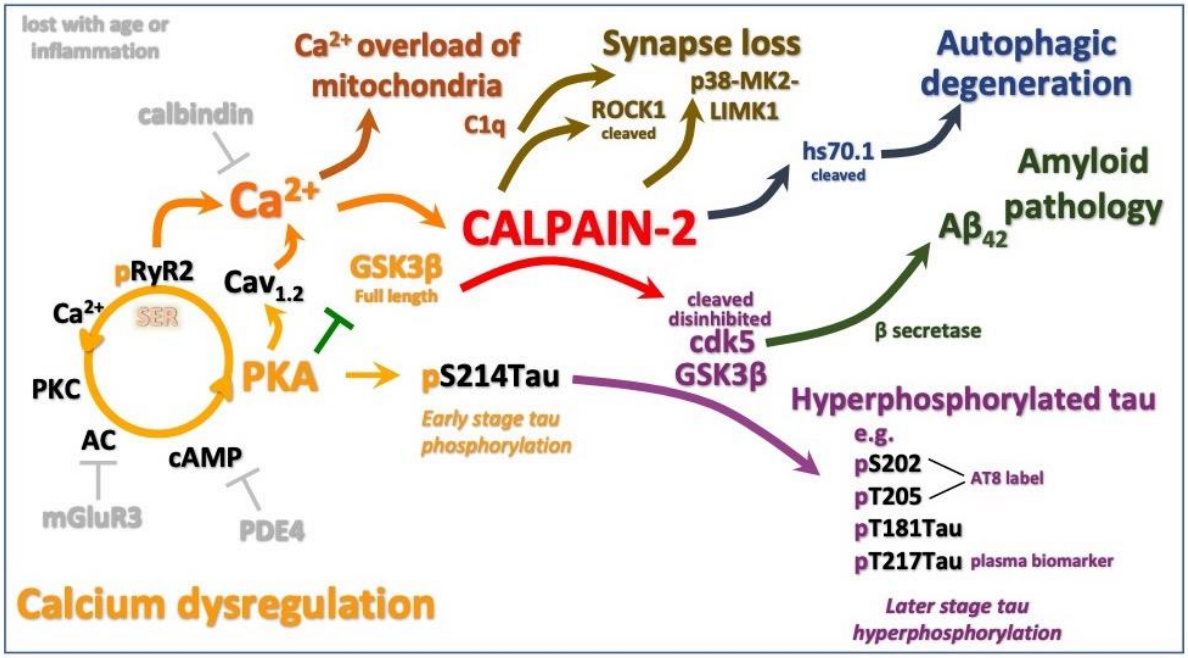

Figure 1. Role of calpain-2 in AD pathology. Dysregulation of feedforward, cAMP-calcium signaling with advancing age and/or inflammation generates high levels of cytosolic calcium, which activates calpain-2. Calpain-2 cleaves and disinhibits GSK3β and cdk5, which hyperphosphorylate tau. Cleaved cdk5 also increases BACE/Aβ production, driving amyloid pathology. Calpain-2 can also drive synapse loss and autophagic degeneration through cleavage of ROCK1 and hsp70.1, respectively, thus worsening multiple pathological events in AD. (Adapted from Arnsten et al, 2021[17]).

Importantly, evidence of calcium leak from the SER through pRyR2 has been documented in brain tissues from patients with sporadic AD, confirming its relevance to the human disease[44,45]. Indeed, some of the earliest evidence of the importance of calcium leak to neural pathology came from Mattson's studies of tissues with PS1 mutations, the most common, and aggressive cause of autosomal dominant, early onset AD. He showed that either a PS1 or PS2 mutation causes dramatic calcium leak from the SER and hypothesized that this would be a significant driver of neuropathology[28,46].

Dysregulated calcium signaling has also been linked to heart failure, where calcium leak from the sarcoplasmic reticulum causes calcium overload of mitochondria in cardiac muscle, driving oxidative stress, which in turn causes more calcium toxicity[47]. Evidence of these pathological events can also be seen in brain of the aged rhesus monkey dlPFC and of AD patients, where calcium overload causes an abnormal mitochondrial morphology called Mitochondria-On-A-String (MOAS)[48,49]. In AD, the activity of the electron transport chain enzyme cytochrome c oxidase is significantly reduced[50,51], ATP production is diminished[52,53], and there is a decrease in both mitochondrial mass and mitochondrial DNA content[54,55]. As mitochondria normally regulate calcium signaling by taking up calcium, mitochondrial dysfunction can further drive calcium dysregulation. When cytosolic calcium levels become abnormally high, they can activate calpain-2, which can cause a large number of pathological events seen in AD brains, as described below, and summarized in Figure 1.

4. Links between calpain and AD

The calcium-dependent proteases, calpains, have long been associated with the pathology of AD[56,57]. Calpains represent a 15-member family of calcium-dependent proteases, and it has been difficult to determine the roles of specific calpain isoforms in the brain in general and in AD in particular. The major calpain isoforms in the brain are calpain-1 and calpain-2, the so-called classical calpains[58]. Recent findings indicate that calpain-1 and calpain-2 play opposite functions in the brain, with calpain-1 being neuroprotective and calpain-2 neurodegenerative[1]. Calpain-2 was also identified as an early component of neurofibrillary tangles in AD using active-site directed antibodies[59]. More recently, hyperactivation of calpain-2 was shown to occur presymptomatically

in a mouse model of AD and to correlate with memory deficits in AD patients[60].

Calpain has long been proposed to participate in the pathology of Alzheimer's disease. It was first speculated that impaired calpain activity could lead to the development of neurofibrillary degeneration and cytoskeletal alterations[61]. A few years later, the idea was advanced that excitotoxity was involved in AD pathology and that activation of calpain-1 was responsible for this phenomenon[62]. Interestingly, Ralph Nixon changed his initial hypothesis and proposed that calpains could promote ß-amyloidogenesis, neurofibrillary pathology and neuronal degeneration[63,64] . In support of this idea, the Nixon group found that calpain-2 specifically is found in association with neurofibrillary tangles in the brains of patients with AD. Other groups have also found that the rise in calpain activity heralds the onset of tau pathology[57].

The Nixon lab tested whether reducing calpain activity would be protective in mouse models, and found that overexpression of the endogenous calpain inhibitor, calpastatin, reduced excitotoxic stress-induced neurodegeneration[65]. The role of cdk5/p25 in the formation of neurofibrillary tangles also supported the involvement of calpain, as calpain is responsible for the cleavage of p35 into p25, the cdk5 activator[66]. These findings led to the suggestion that calpain inhibitors could be used as neuroprotective drugs for the treatment of various neurodegenerative disorders, including AD[67-69]. A recent review summarized a whole body of evidence linking calpain to AD, including the roles of calpain in both Aß and tau pathology, as well as in autophagy impairment[57]. It also reviews many studies showing that a variety of calpain inhibitors provided beneficial effects on a range of pathological parameters, including learning and memory, Aß accumulation and tau hyperphosphorylation in various animal models of AD. Depletion of the endogenous calpain inhibitor, calpastatin, resulting from the activity of caspases and calpain, has been shown to produce calpain-2 activation and tau and neurofilament hyperphosphorylation[70]. All these findings led Abbvie to conduct a Phase I clinical trial with a non-selective calpain inhibitor, ABT-957, aka Alicapistat, for Alzheimer's disease. The study tested the effects of the calpain inhibitor in both healthy and MCI patients, with twice daily doses and for 12 weeks. However, the study was terminated due to lack of pharmacodynamic effects, assessed by changes in REM sleep, suggesting that the inhibitor did not reach high enough levels in the brain[71].

In addition to driving the hyperphosphorylation of tau, activated calpain increases BACE and Aβ levels via cleavage of cdk5[72], and drives autophagic degeneration through the activation of heatshock protein 70.1 (Hsp70.1)[73], as summarized in Figure 1. As neurons die from autophagic degeneration in AD, and not by apoptosis, this latter finding suggests that calpain-2 may simultaneously drive tau pathology and autophagic degeneration within the same neurons. This would be consistent with Yamashima's "calpain-cathepsin hypothesis" for AD. In this model, calpain-mediated cleavage of Hsp70.1 results in the permeabilization of lysosomes and the leakage of cathepsin in the cytoplasm[74].

In most of these studies, there is little, if any, discussion regarding the respective roles of calpain-1 and calpain-2, the major calpain isoforms ubiquitously expressed in the brain. Both calpain-1 and calpain-2 appear to be elevated in AD brains and herald the rise in tau pathology[75], but only calpain-2 is localized with neurofibrillary tangles (NFTs) and pretangles[59]. Importantly a recent study reported specific hyperactivation of calpain-2 in in both a mouse model of AD and in cortical tissues from post-mortem AD patient brains[60]. Elevated calpain-2 was observed in presymptomatic 1 month-old APPswe/PS1ΔE9 mice and persisted up to 10 months of age. In humans, elevated calpain-2 activity was significantly correlated with cognitive impairment. Interestingly, while calpain-2 activity was positively correlated with Aß load, there was no correlation between calpain-2 activity and hyperphosphorylated tau[60]. The authors postulated that elevated calpain-1 activity might happen later in the disease progression and could be responsible for tau pathology. Clearly, more work remains to be done to clarify the respective roles of calpain-1 and calpain-2 on the various pathological manifestations of AD.

As mentioned above and by many reports[76], calpain drives a spectrum of AD-related pathologies, and in particular, tau hyperphosphorylation by cleaving, and thus disinhibiting, kinases central to tau hyperphosphorylation, i.e., GSK3β and cdk5. GSK3β and cdk5 hyperphosphorylate tau at key sites, including pS202 and pT205 (labeled by AT8), and pT181Tau and pT217Tau, which are important, emerging fluid biomarkers for AD pathology, including pT217Tau in plasma[77,78]. Early stage tau phosphorylation at S214 by PKA primes tau for

subsequent hyperphosphorylation by GSK3β[79], and thus creates a reservoir of primed phosphorylated tau (pTau) species (Fig. 1). Calpain-2 cleavage of GSK3β appears to be a critical step, as it removes the site where PKA normally inhibits GSK3β[80,81], and thus allows GSK3β to phosphorylate tau without any regulation. Calpain-2 also selectively cleave and deactivate the tyrosine phosphatase, PTPN13, which can indirectly promote tau phosphorylation, and produces a stable breakdown product, P13BP, that can be measured in CSF and plasma[82,83].

Another important aspect of the role of calpain in AD pathology is the putative role of calpain in generating tau fragments that might seed the formation of neurofibrillary tangles[76,84,85]. The issue of whether calpain-mediated tau cleavage results in neurotoxicity remains debated. Results in Drosophila convincingly showed that calpain-mediated generation of a 17 kD fragment of tau was responsible for neurotoxicity[86]. On the other hand, Garg et al.[87] concluded that the 17 kD tau fragment was not a mediator of Aß toxicity. More recently, aggregates of the 17 kD tau fragment (also referred to as tau_{45-230}) were found in AD brains and could be internalized and induce neurodegeneration in hippocampal neurons[88]. However, there is a general agreement that calpain cleavage and activation of GSKß and cdk5 could drive AD pathology, and thus this would strongly support the idea that a selective calpain-2 inhibitor could have beneficial effects in AD and may preserve the beneficial effects of calpain-1 needed for housekeeping functions and neuroplasticity.

5. Calpain-2 and neurodegeneration

Calpain activation has been implicated in neurodegeneration since the early 80s, and most reviews written on calpains all mentioned the role of calpains in neurodegenerative processes[89-92]. However, very few studies have explored the specific contributions of calpain-1 and calpain-2 in neurodegeneration. Since 2012, our laboratory has focused on evaluating the contributions of calpain-1 and calpain-2 in both synaptic plasticity and neurodegeneration. We showed that calpain-2, but not calpain-1, activation was responsible for NMDA-induced excitotoxicity through the activation of the striatal-enriched tyrosine phosphatase (STEP) in primary neuronal cultures[93]. A similar study indicated that knock-down of calpain-2, but not calpain-1, increased neuronal survival following NMDA treatment of cultured hippocampal neurons [94]. Because calpains cleave a large number of proteins involved in neurodegeneration[95], it has been difficult to determine under various experimental conditions which of them are responsible cell death. Importantly, we found that calpain-2 is associated with a multi-protein complex including extrasynaptic NMDARs, which have been shown to be involved in neuronal death[96]. In particular, NR2B subunits are enriched in extrasynaptic NMDARs[97], and their activation leads to calpain-2-mediated cleavage of STEP and to neuronal death[98]. NR2B also interacts with RasGRF1, which leads to ERK activation[99], calpain-2 phosphorylation and prolonged calpain-2 activation[100]. Numerous studies have shown that calpain cleaves (STEP), generating inactive fragments, resulting in the activation of p38 and downstream cell death signaling pathways[98,101]. While this pathway is activated following acute brain injury, it might not be the one involved in chronic neurodegenerative diseases, as neurons appear to die by autophagy and not apoptosis.

As discussed above, over the last 20 years, Yamashima has developed the calpain-cathepsin hypothesis to account for several features of neuronal death in Alzheimer's disease[74,102-104]. A main feature of this hypothesis is the truncation of carbonylated Hsp70.1 by calpain, leading to the destabilization of lysosomal membranes and the release of cathepsins in neuronal cytoplasm. Incorporated in this hypothesis is the concept that oxidative stress, which has often been associated with AD[105], could stimulate the formation of carbonylated Hsp70.1, and calpain activation through disruption of mitochondrial function. Reactive oxygen species (ROS), which accumulate as a result of mitochondrial dysfunction, have been shown to activate calpain and in particular, calpain-2 in several types of cells and under a variety of experimental conditions[106-108]. There is therefore a direct link between calpain-2 activation, lysosomal dysfunction and neuronal death.

6. Conclusions

Accumulating evidence indicates that overactivation of calpain-2 plays a critical role in the initiation and progression of AD pathology. Calcium dysregulation within glutamatergic neurons in the limbic and association cortices with advancing age may activate calpain-2 to spur AD pathology, including Aß generation, tau hyperphosphorylation and autophagic degeneration. As calpain activation heralds tau pathology, and calpain-2 is specifically associated

with neurofibrillary tangles in AD brain, calpain-2 may be an important therapeutic target. Early treatment with a selective calpain-2 inhibitor may therefore provide a real preventive treatment for AD, leaving the beneficial effects of calpain-1 intact. This strategy may allow a treatment with a very low side effect profile, necessary for early, long-term treatment.

A challenge of preventive trials is how to measure early indices of therapeutic efficacy, e.g., at an age prior to the formation of fibrillated Aß and pTau species measured by PET imaging. Plasma and CSF biomarkers may be a fruitful direction forward, given the recent successes in this arena. The effects of calpain-2 inhibition on tau and amyloid pathology could be assessed with plasma and CSF measures of pT217Tau, and CSF assays of Aß ratios, which are altered at very early stages. The efficacy of a calpain-2 inhibitor on calpain-2 activity could also be assessed using a new blood biomarker, consisting of the calpain-2-mediated fragment of the tyrosine phosphatase, PTPN13, and referred to as P13BP, which has been found to be elevated in postmortem samples from AD patients[82], and thus may be used to track drug efficacy in addition to plasma and CSF markers of pT217Tau and Aß ratios. These methods may allow the development of treatments that could actually prevent AD by reducing pathology at its earliest stages. Furthermore, new drug delivery devices are targeting brain more specifically by using intranasal delivery[109,110]. Such an approach would mitigate the potential harmful peripheral effects of a chronic treatment with a selective calpain-2 inhibitor.

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