
Using Proteomics to Understand Alzheimer's Disease Pathogenesis

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Abstract: Our current understanding of the molecular changes that drive Alzheimer's disease (AD) pathogenesis is incomplete. Unbiased, mass-spectrometry-based proteomic studies provide an efficient and comprehensive way to quantitatively examine thousands of proteins at once using microscopic amounts of human brain tissue. Recently, the number of proteomic studies that examine protein changes in AD brain tissue has been increasing. This chapter reviews the different proteomic approaches currently being used to identify pathological protein changes in AD brain tissue including bulk tissue studies that examine protein changes throughout the progression of AD, studies of the insoluble proteome in AD, studies using proteomics to examine selective vulnerability in AD, studies of the amyloid plaque and neurofibrillary tangle proteome, studies of the synaptic proteome, and studies of the interactome of beta amyloid and tau. Combined, these complementary proteomic approaches provide increased understanding about the protein changes that occur in the AD brain. Results from these proteomic studies provide an excellent resource for future hypothesis-driven targeted studies and will help identify new biomarkers of disease and new drug targets for AD.

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INTRODUCTION

Alzheimer's disease (AD) is a complex, multifactorial disease. Various genetic, environmental, and lifestyle risk factors have been associated with the development of AD; however, none of these have been shown to definitively cause late onset AD (1). AD is diagnosed at autopsy by the presence of characteristic neuropathology: amyloid plaques and neurofibrillary tangles (NFTs), which primarily consist of aggregated beta amyloid ($A\beta$) and hyperphosphorylated tau, respectively (2). The development of these neuropathological lesions is associated with increased neuroinflammation, synaptic loss, neurodegeneration, and ultimately the development of cognitive impairment that clinically characterizes AD. Imaging and biomarker studies suggest that AD begins ~20 years before the development of dementia, resulting in a long preclinical stage of disease before clinical symptoms are apparent (3).

There are still significant gaps in our understanding about the molecular mechanisms that underlie the pathogenesis of AD. For example, we do not know what causes AD, what factors drive the development of neuropathology, what factors cause the development of cognitive impairment, or what factors are responsible for the considerable heterogeneity in the rate of progression in people with AD. A greater understanding of all of these factors is essential for the development of effective therapeutics and discovery of new biomarkers for AD. New therapeutics are particularly needed for AD as the previous record for AD clinical trials has been very poor: 99.6% of AD clinical trials have failed, and currently, no disease-modifying treatment is available. This high failure rate has been attributed to various factors including starting treatment too late in the disease process, having the wrong drug targets, or relying too heavily on results from preclinical studies that use animal models of AD that poorly reflect human disease (4–6).

THE BENEFITS OF USING PROTEOMICS TO STUDY PROTEIN CHANGES IN AD BRAIN TISSUE

Traditionally, studies examining the molecular mechanisms that drive AD pathogenesis have used a targeted, hypothesis-driven approach that focuses on select proteins of interest. This approach has uncovered many of the major players involved in AD pathogenesis, most notably identifying beta amyloid ($A\beta$) as the major protein present in amyloid plaques (7, 8), identifying tau as the major protein present in NFTs (9), and identifying apolipoprotein E (apoE) as the most significant genetic risk factor for late onset AD (10, 11). However, using a targeted approach precludes the discovery of novel disease-associated proteins and limits the ability to understand these protein changes in the broad context of AD.

Unbiased, hypothesis-free “omics” studies such as genomic, transcriptomic, epigenetic and proteomic studies offer a comprehensive, highly efficient way to identify genes or proteins that are involved in the pathogenesis of AD. The high-throughput nature of “omics” studies means that they can be performed using microscopic amounts of human tissue samples, which are essential to study when examining diseases that are unique to humans, such as AD (12). Genomic and epigenetic studies have successfully identified new genetic risk factors for late-onset AD and have provided the basis for new hypothesis-driven studies examining how these genetic variants and epigenetic changes are involved in AD (13–16). Unbiased, mass-spectrometry-based proteomic studies of human AD brain tissue are essential to complement these genomic studies, particularly given that proteins are the druggable targets in AD. Furthermore, there is a poor correlation between RNA expression and protein levels in AD brain tissue; therefore, transcriptomic or genomic studies do not provide a complete picture of the pathogenic changes in the AD brain (17). Using mass-spectrometry-based proteomics to study the pathogenesis of AD has many advantages including the following: thousands of protein differences can be quantified simultaneously using microscopic amounts of brain tissue, the unbiased nature of these studies permits the discovery of novel proteins involved in AD pathogenesis, and proteomics can detect post-translational modifications on proteins (e.g. phosphorylation, oxidation, and ubiquitination) that are known to have an important pathological role in AD. The large amount of data generated in proteomic studies provides a comprehensive, bird's eye view of all protein differences that occur in AD, which can provide insight into the molecular mechanisms that cause AD at a network/systems level, which is particularly useful when studying complex diseases like AD (18, 19). Mass-spectrometry-based proteomic studies have been limited in the past by technical and financial constraints; however, these factors have recently become less restrictive, and consequently, the number of proteomics studies using AD brain tissue has increased.

AD PROTEOMIC STUDIES USING BULK TISSUE HOMOGENATES

The majority of proteomic studies examining AD brain tissue have defined the proteomic changes between AD and age-matched, cognitively normal controls using bulk tissue samples. In these studies, proteomics is used to compare protein expression between AD and controls in brain homogenate, usually limited to one vulnerable brain region. Early liquid chromatography-mass spectrometry (LC-MS) studies generated preliminary findings about protein differences between AD and control brains, but were typically restricted by small sample sizes and therefore struggled to detect protein differences after correcting for multiple comparisons (20–26). More recent studies have included a larger number of samples, which are consequently sufficiently powered to detect hundreds of protein differences in AD brains (17, 27–34). Encouragingly, meta-analysis of these recent studies shows that many of the significantly altered proteins in AD brains are consistent, leading to increased confidence that these altered proteins are relevant to the pathogenesis of AD.

The most comprehensive studies have been conducted by researchers at Emory University, USA (17, 30, 31). Their studies primarily examined protein differences in the frontal cortex throughout the progression of AD, specifically comparing protein levels in advanced AD, asymptomatic AD (also referred to as preclinical AD), and age-matched cognitively normal subjects. Combined, these studies identified hundreds of protein differences present at different stages of AD. They found that the number of protein differences steadily increased with disease progression, suggesting that the number of protein differences is reflective of increased dysfunction involving more pathways as AD progresses. Their analysis allowed the identification of subsets of proteins that were exclusively altered in the symptomatic phase of AD and those that were altered prior to the onset of clinical symptoms. For example, they showed that proteins involved in synaptic function and synaptogenesis progressively decreased throughout AD, starting before clinical symptoms were present. They also showed that altered RNA metabolism and increased inflammation were present in AD brains in the earliest stages of disease prior to cognitive impairment. In contrast, astrocyte and microglia proteins increased in late stage AD and showed a strong correlation with the number of NFTs present. A consequent study by the same group specifically focused on the protein differences present in AD cases stratified by ApoE genotype (33). ApoE is the major genetic risk factor for late onset AD (35, 36). The three alleles of ApoE (apoE2, apoE3, and apoE4) confer different risk for AD: apoE4 increases risk for AD and apoE2 decreases risk for AD. Their proteomic results suggested that apoE may confer risk in AD through a combination of effects on inflammation, metabolism, and cerebral vasculature, and using their proteomic approach, they were able to pinpoint the specific proteins involved (33).

One important factor to be mindful of when interpreting and comparing proteomic studies is the type of tissue lysis method used prior to mass spectrometry. Different lysis methods enrich for different populations of proteins or even different pools of the same protein. For example, soluble and insoluble forms of the same protein may require different lysis methods for detection. Therefore, the use of various lysis methods can complicate meta-analysis of multiple proteomic studies as the same proteins are not always detected by each lysis method. However, one advantage of using varied lysis methods is that combined analysis of proteomic studies that use various lysis methods provides a richer view of molecular changes in the AD brain. For example, some studies have specifically examined differences in the insoluble proteome in AD (21, 25, 30, 37), which enriches for proteins that are associated with the insoluble plaques or NFTs in AD, as well as other proteins that are independently prone to insolubility in the AD brain. Of these studies, Hales et al. provide the most comprehensive analysis of insoluble protein changes in AD (30). Interestingly, they showed that the number of differentially expressed proteins in the insoluble fraction increased with disease severity and that many of these insoluble proteins were involved in mitochondrial function, which is known to be decreased in AD (30). Other recent studies have used proteomics to answer specific questions about which proteins are primarily affected by post-translational modifications in AD. Two recent studies have used enrichment strategies to identify all proteins that are phosphorylated and ubiquitinated in AD (38, 39). These studies showed that the number of ubiquitinated proteins was much higher in AD brains than in control brains, which is consistent

with the accumulation of insoluble and misfolded proteins during AD and reflects the proteolytic stress present in AD (38). Examination of phosphorylated proteins confirmed that tau was the most highly phosphorylated protein in AD in comparison to controls and also identified an additional 142 proteins that were phosphorylated to a greater extent in AD brains (39).

THE USE OF PROTEOMICS TO UNDERSTAND SELECTIVE VULNERABILITY IN AD

One of the most striking features of AD is that specific brain regions are particularly vulnerable to the development of amyloid plaques, NFTs, and neurodegeneration, while other regions are comparatively resistant to pathology. Why this occurs is still unknown. However, various factors have been proposed to contribute to vulnerability including: gene expression, long axonal projections or large neuronal size, being an excitatory neuron, containing low levels of calcium buffering proteins, or containing high levels of metastable subproteome proteins that are prone to aggregate in times of stress (40–42). Defining a particular brain region as vulnerable or resistant in AD can be complex, as it depends on the neuropathological factor you use to define vulnerability. This is important because some brain regions are preferentially vulnerable to developing amyloid plaques, while others are preferentially vulnerable to developing NFTs or neurodegeneration, and the presence of these different types of neuropathology does not always correlate. In general, regions that are particularly vulnerable to the development of AD include the hippocampus, entorhinal cortex, basal forebrain, and locus coeruleus. Comparatively resistant regions include the cerebellum and occipital cortex.

Transcriptomics studies suggest that there is likely a distinct protein signature of vulnerable neurons in AD (43); however, this has not yet been comprehensively examined at the protein level. Proteomic studies of selective vulnerability in AD are complex as additional variables need to be considered in their experimental design. For example, basal protein differences between different brain regions have to be accounted for when interpreting protein differences that appear to be associated with increased vulnerability to AD. This is particularly important when comparing brain regions that are morphologically different such as the cerebellum and the hippocampus. Disparate basal protein expression between brain regions complicates interpretation of results as protein differences could be due to either basal brain region differences or AD associated differences. Accounting for these variables is possible, but ultimately results in large, complex studies that require a large number of samples to perform all analyses with sufficient power.

The majority of bulk tissue proteomic studies have only analyzed one or two brain regions, usually focusing only on vulnerable brain regions, meaning that they cannot be used to examine the protein changes associated with selective vulnerability in AD. Two recent studies have aimed to fill this knowledge gap. Xu et al. (44) performed the most extensive analysis of proteomic changes in the AD brain that are associated with selective vulnerability. They compared protein expression in three highly affected regions (hippocampus, entorhinal cortex, and

cingulate gyrus), two lightly affected regions (sensory cortex and motor cortex), and one comparatively unaffected region (cerebellum). As expected, the majority of protein differences between AD and control brains were observed in the highly affected regions, and these protein changes were reflective of increased innate and adaptive immune responses in the brain and increased apoptosis. The fewer protein differences in the lightly affected regions appeared to be reflective of early stage pathology, suggesting that the same molecular changes that drive pathogenesis of AD eventually spread to these lightly affected regions. Intriguingly, the cerebellum actually showed a large number of protein differences between AD and controls, even more so than the lightly affected regions. However, these protein differences appeared to be reflective of potentially protective molecular changes such as increased expression of proteins associated with growth factors, increased oxidative defense proteins, and decreased transfer RNA synthetases. Mendonca et al. (45) also performed a comprehensive study looking at the proteomic differences in brain regions preferentially vulnerable to tau pathology in AD. They compared the proteome in AD and controls in two brain regions that are highly vulnerable to the development of NFTs (parahippocampal cortex and entorhinal cortex) and two brain regions that are moderately vulnerable to the development of NFTs (temporal cortex and frontal cortex). In doing so, they generated a complex dataset that will be useful for future data mining studies examining the protein changes associated with tau pathology in AD. In the future, expanding these studies to include comparisons between multiple brain regions at multiple stages of AD will be useful for defining the protein differences that underlie selective vulnerability in AD and to definitively determine whether mildly affected regions show molecular changes that are similar to those in early stage AD.

PROTEOMICS OF NEUROPATHOLOGICAL FEATURES PRESENT IN AD

Other groups, including my own, have recognized the importance of performing localized proteomic studies that specifically focus on disease-associated neuropathological features or specific cell populations. Using a localized approach that focuses specifically on areas highly affected by disease has the potential to reveal protein differences between AD and controls that are particularly relevant to pathogenesis. We have focused our efforts on using a localized proteomics approach to determine the proteome of amyloid plaques, NFTs, and vulnerable neurons. In this approach, neuropathological features or vulnerable neuron populations are microdissected from sections of human brain tissue, and their protein composition is analyzed using mass spectrometry (46–48). A key advantage of our approach is that it can be performed using formalin-fixed paraffin-embedded (FFPE) tissue. This is important because the majority of human tissue specimens available for research are FFPE blocks of tissue that are collected and used during autopsy. Therefore, developing a method that is compatible with FFPE tissue greatly increases the feasibility of human tissue studies, particularly those using rare or unique cases. A second key advantage of our method is that it can be performed using microscopic amounts of tissue. We have successfully performed

proteomics using as little as 1.5 mm² of tissue, which is the equivalent of approximately 550 amyloid plaques or 4000 NFTs. This number of plaques and tangles can typically be collected using <4 tissue sections, showing just how little tissue is required for these studies. But the most important aspect of our approach is that we can quantify over a 1000 proteins at once using these microscopic tissue samples, which therefore provide a comprehensive analysis of the proteins that are associated with neuropathological features in the AD brain and proteins that are associated with selective vulnerability of specific neuronal populations. For example, we showed that amyloid plaques consistently contained hundreds of proteins in addition to A β and that many of these were novel proteins that had not previously been associated with AD (49, 50). Importantly, we also showed that the protein composition of amyloid plaques was significantly different in people with rapidly progressive AD in comparison to typical sporadic AD, suggesting that different molecular mechanisms may underlie plaque development in different subtypes of disease. We also recently examined the proteome of NFTs and identified over 500 proteins in NFTs in addition to tau, many of which were novel (51). These examples show the power of an unbiased localized proteomics approach to efficiently identify hundreds of proteins that are associated with amyloid plaques or NFTs. These findings can be used as the basis for future targeted studies that aim to determine the mechanistic involvement of these proteins in AD.

One example protein that we discovered using proteomics and have since followed up on in a targeted study is secernin-1. Very little is known about the function of secernin-1, and no study has previously associated secernin-1 with AD. We identified secernin-1 as a novel amyloid plaque protein in our previous proteomic study of amyloid plaques (49). We have since performed a comprehensive neuropathological study of secernin-1 accumulation in early and late stage AD (52). Surprisingly, we found that secernin-1 abundantly and specifically accumulated in NFTs in AD and that its presence in amyloid plaques was limited to accumulation in the dystrophic neurites present in neuritic plaques. Co-immunoprecipitation showed that secernin-1 directly interacted with phosphorylated tau in AD brains, suggesting that it could have an important role in mediating the toxic actions of tau in AD. Intriguingly, secernin-1 colocalized with phosphorylated tau aggregates only in AD and not in other neurodegenerative diseases that also show the presence of aggregated phosphorylated tau including Pick's disease, progressive supranuclear palsy, and corticobasal degeneration. This suggests that secernin-1 could be a new potential biomarker that discriminates between AD and other tauopathies. These results show that localized proteomics studies are capable of identifying new biomarkers of disease and new potential drug targets. Secernin-1 is just one example protein from a list of many new potential candidates that we have identified in our proteomics studies that can be examined in future mechanistic studies.

A small number of other groups have also used a similar localized proteomics approach to study the proteome of neuropathological features or vulnerable cell populations/brain regions in AD. Two small studies have examined the proteome of human amyloid plaques (53, 54), and two small studies have examined the proteome of human NFTs (55, 56). Three other studies have examined the proteome of cerebral amyloid angiopathy (CAA), which is present when A β pathologically accumulates in blood vessels (57–59). Combined, all of these studies provide preliminary data that have hinted at new proteins that are associated

with these neuropathological features; however, the small number of cases included in these initial studies means that further studies are needed to provide a comprehensive understanding of the proteome of amyloid plaques, NFTs, and CAA. Future studies examining larger numbers of cases that are stratified by AD subtype will be very informative in helping to identify proteins that have a particular interaction with neuropathological features and to determine whether proteins associated with these neuropathological features are different between subtypes of AD.

PROTEOMICS OF SYNAPTIC FRACTIONS IN AD

Synapse loss is an early feature of AD that closely correlates with cognitive impairment (60–63). Understanding the synaptic protein changes in AD could help us understand what is driving this process. Multiple studies have been completed that have analyzed the proteome of synaptosomes and post-synaptic density in control human brains, which have been nicely combined in a recent meta-analysis (64). However, a comprehensive analysis of the synaptic proteome in AD has not yet been performed. Preliminary results have been generated that analyzed the proteome of synaptosomes (65, 66) or post-synaptic density fractions (67). However, the small sample sizes used in these studies (between $n = 2$ and $n = 6$) mean that their findings are not yet definitive. Other studies have used their bulk tissue homogenate results to look specifically at synaptic protein changes (34, 68); however, these results could potentially miss differences in low abundance synaptic proteins. To date, all studies examining synaptic protein differences in AD have compared advanced AD and controls. Given that synaptic loss is an early feature of AD, it would be particularly useful to determine the protein changes that contribute to synapse loss in either mild cognitive impairment or preclinical AD. Larger studies examining differences in the synaptic proteome in early AD are currently ongoing in the field, and these will likely provide a greater overview of the specific protein changes that contribute to synaptic loss in AD. Future results detailing the synaptic protein differences in AD will be very interesting given that it has been recently suggested that synaptic proteins in the cerebrospinal fluid may also be excellent new biomarkers for early AD (64, 69).

ANALYSIS OF THE A β OR TAU INTERACTOME IN AD

Another useful proteomics approach to study AD pathogenesis is using affinity purification-mass spectrometry to identify the proteins that interact with toxic A β or tau species in AD. In this approach, particular species of A β or tau are isolated from human brain samples using antibodies. Proteins that interact with A β or tau are isolated at the same time, and mass spectrometry is used to identify these interacting proteins. This is a powerful approach because it allows the efficient and comprehensive examination of all proteins that interact with A β or tau in an unbiased manner. It can also determine which proteins interact with particular species A β or tau, which is important as some species are more toxic than others.

Results from these studies have the potential to increase our understanding about how A β and tau are involved in the pathogenesis of AD and could lead to the discovery of new drug targets.

Despite A β being the predominant focus of AD research for decades, there is a surprisingly limited number of studies that have used affinity purification mass spectrometry to examine the A β interactome. One possible reason for this is that it is difficult to find an appropriate antibody that specifically recognizes A β and not its longer precursor protein (amyloid precursor protein; APP). Accordingly, a number of studies have instead examined the interactome of APP in mouse brain tissue (70, 71) and in cells expressing human APP (72, 73). However, despite this limitation, there have been two recent studies that have developed alternative ways to examine the A β interactome. The first study isolated aggregated A β complexes from human brain samples using a non-specific A β antibody (that also recognizes APP), but limited their downstream proteomic analysis to only those proteins present in the insoluble fraction, with the assumption that the resulting interacting proteins were limited to those present in insoluble A β -containing aggregates rather than APP (37). The second study used a more traditional approach of binding recombinant monomeric A β 42 or oligomeric A β 42 to beads that were then used to pull down interacting proteins from human brain samples (74). Combined, these studies identified over 100 proteins that interact with A β , including some proteins that preferentially interacted with oligomeric A β in comparison to monomeric A β . However, more studies are needed in the future that compare the interactome of different A β species (such as A β 40, A β 42, and pyroglutamate modified A β) and that determine the endogenous pathological interactions present in AD brain tissue, as these may be different than those present in artificial *in vitro* experiments.

To date, all studies examining the tau interactome have used total tau antibodies that identify proteins that interact with all tau species. Two studies have examined using human brain tissue (37, 75), while others have examined tau interactors in mouse brains (76–79) and in cells expressing human tau (80). These studies found that different isoforms or domains of tau regulate different protein interactions, identified the major protein families that tau preferentially binds to, and identified new potential drug targets for preventing tau toxicity. However, one limitation of these studies is that using a total tau antibody results in the identification of all proteins that interact with both physiological and pathological tau in the brain, therefore making it difficult to determine which interactions are specific to the pathological phosphorylated tau species present in AD brains. Therefore, we have recently completed the first study of the phosphorylated tau interactome in human AD brain samples (51). Our results showed that phosphorylated tau in AD brains preferentially interacted with neuronal proteins, which is consistent with the intraneuronal location of phosphorylated tau in AD. We found that phosphorylated tau particularly interacted with proteins associated with two of the main protein degradation systems in the cell: the ubiquitin–proteasome system and the phagosome–lysosome system. The specific proteins involved suggested that phosphorylated tau may be potentially interfering with degradation of proteins by the proteasome and may contribute to lysosomal dysfunction in AD via interference with vacuolar ATPase proton pumps that are responsible

for acidification of lysosomes. Impairment of both of these processes has been previously associated with AD (81–83); however, this is the first study to show that tau may be involved in this process. This is an example of the informative nature of interactome studies, showing that they can provide a complete and unbiased overview of the pathogenic brain changes that occur in AD that are directly linked to a specific toxic protein species.

Going forward, performing these studies in a systematic manner that directly compares the interactome of multiple A β or tau species will help determine which protein interactions are particularly important for disease progression. Determining these key interactions that drive toxicity and that drive the formation of plaques or NFTs will help identify new potential drug targets for AD.

CONCLUSION

In conclusion, proteomics studies using human tissue are very useful for increasing our understanding about the pathogenesis of AD. The combined proteomic results from studies described above provide a powerful resource for generating new hypotheses about the cause of AD. Unbiased, proteomic studies using AD brain tissue have been previously limited by concerns about cost, technical limitations, and the assumption that very large sample sizes are required to counteract the large inter-patient variability in AD. However, recent studies have shown that hundreds of significant protein differences can be detected using sample sizes as low as 5 when comparing AD and controls. Larger sample sizes appear to be required when comparing different stages of AD (e.g., preclinical AD vs. advanced AD) or different subtypes of disease (e.g., rapidly progressive AD vs. sporadic AD); however, even in these studies, 20 samples/group are sufficient to identify hundreds of protein differences between groups. These results show that discovery proteomic studies using AD brain tissue are feasible. Importantly, meta-analysis of proteomics studies using AD brain tissue shows that many altered proteins in AD brain tissues are consistent between studies, therefore also validating these findings.

Going forward, it will be useful to expand the scope of these previous studies. Focusing on localized proteomics changes, either in neuropathological features, vulnerable neuron populations, or synaptic fractions, has the potential to greatly increase our understanding about what protein changes drive the development of neuropathology or neurodegeneration in these particularly affected regions. Systematic examination of the proteins that interact with specific species of A β or tau will help identify how these two proteins cause toxicity in AD. Results from localized or interactome studies have the potential to identify new drug targets or biomarkers of disease that are directly associated with AD neuropathology. Determining the protein changes that occur throughout the progression of AD is also particularly important to examine in future studies: the ideal drug targets for AD are pathological changes that occur in the earliest stages of disease; therefore proteomic studies that characterize protein changes in preclinical AD or mild cognitive impairment should be a priority.

Combined, proteomic studies are capable of providing a roadmap of protein changes that are associated with AD. These studies pinpoint the protein networks

that are most involved in disease as well as the specific proteins that are involved. Overall, these studies provide an excellent resource for future hypothesis-driven targeted studies that will hopefully help identify new biomarkers of disease and will help in the development of new drugs for AD.

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