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Review

Phosphoproteomics of Alzheimer disease brain: Insights into altered brain protein regulation of critical neuronal functions and their contributions to subsequent cognitive loss☆

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ABSTRACT

Alzheimer disease (AD) is the major locus of dementia worldwide. In the USA there are nearly 6 million persons with this disorder, and estimates of 13–20 million AD cases in the next three decades. The molecular bases for AD remain unknown, though processes involving amyloid beta-peptide as small oligomeric forms are gaining attention as known agents to both lead to oxidative stress and synaptic dysfunction associated with cognitive dysfunction in AD and its earlier forms, including amnestic mild cognitive impairment (MCI) and possibly preclinical Alzheimer disease (PCAD).

Altered brain protein phosphorylation is a hallmark of AD, and phosphoproteomics offers an opportunity to identify these altered phosphoproteins in order to gain more insights into molecular mechanisms of neuronal dysfunction and death that lead to cognitive loss. This paper reviews what, to this author, are believed to be the known phosphoproteomics studies related to in vitro and in vivo models of AD as well as phosphoproteomics studies of brains from subjects with AD, and in at least one case in MCI and PCAD as well. The results of this review are discussed with relevance to new insights into AD brain protein dysregulation in critical neuronal functions and to potential therapeutic targets to slow, or in favorable cases, halt progression of this dementing disorder that affects millions of persons and their families worldwide.

1. Introduction

Regulation of protein function most often occurs by some type of post-translational modification (PTM), the principal one being protein phosphorylation [1]. Two major types of kinases, serine/threonine- and tyrosine-kinases, catalyze phosphorylation of serine and threonine and tyrosine residues, respectively. Such protein PTMs can have significant effects on protein structure, hence, regulating the protein function. For example, if a Ser or Thr located near a Glu or Asp residue in the protein, the negative charges of the phosphate group and the negatively charged amino acids will drive the groups apart, markedly changing the localized structure of the protein (Fig. 1). In contrast, if Ser or Thr near a Lys or Arg group is phosphorylated, the negatively charged phosphate group will be attracted to the positive amino acids, again significantly changing the localized structure of the protein (Fig. 1). In the case of Tyr phosphorylation, Tyr kinases, either alone or as a receptor-activated Tyr kinase, are often involved in processes associated with signal transduction following ligand binding to a specific receptor [2]. Any modification of the phenolic ring of Tyr that sterically blocks access of the Tyr OH group would lead to decreased receptor-tyrosine kinase signaling, clearly detrimental to cell survival. Along with kinases, protein phosphatases that remove protein-bound phosphate groups are involved in phosphorylation of proteins, and, consequently, also contribute to regulation of protein function [3].

About 500 kinases and 100 phosphatases are in the human genome [4]. Their complex interactions among various kinases and phosphatases regulate the dynamics of critical cellular processes [5]. Often these kinases and phosphatases are themselves regulated, certainly by other kinases and phosphatases, but also for example by the cis-trans prolyl isomerase, Pin1 [6,7], which in Alzheimer disease (AD) is oxidatively dysfunctional [8,9]. This, together with differential expression or post-translational modification of kinases and phosphatases, cause loss of regulation of these kinases and phosphatases, resulting in compromised cellular processes. Such is the case in AD brain as discussed further below based on results of phosphoproteomics.

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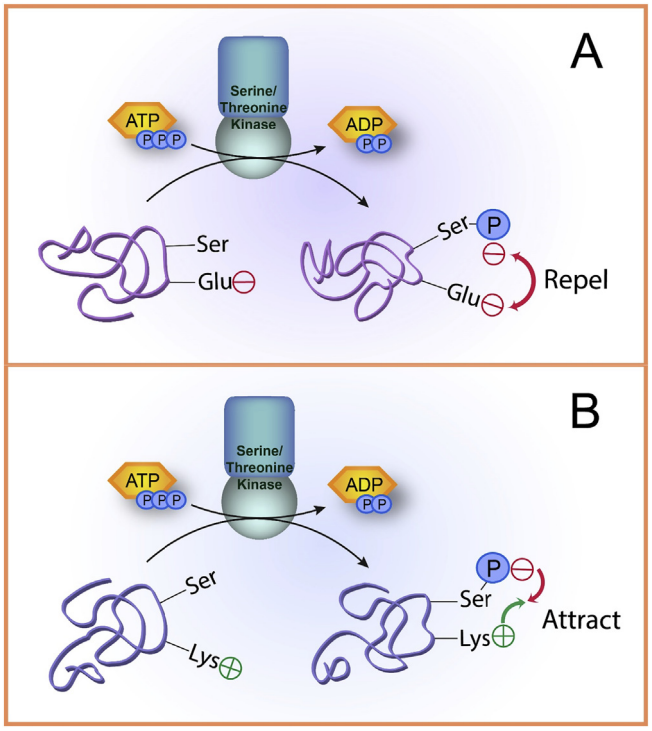

Fig. 1. Phosphorylation of Ser, Thr, or Tyr residues on proteins regulates the function of these proteins due to electrostatic effects. In most cases in biochemistry, function changes if structure changes. Upper figure: Phosphorylation of Ser located near a negatively charge amino acid (shown here Glu) puts two negatively charged species in close proximity that leads to their repulsion and consequent alterations of the 3-dimensional structure of the protein and consequent change in function. Lower figure: Similar considerations apply but in this case phosphorylation occurs in close proximity to a positively charged amino acid (shown here Lys) that causes attraction of these two oppositely charged residues, again with changes in the 3-dimensional structure of the protein and consequent change in function.

2. Alzheimer disease

The major age-related neurodegenerative disorder in humans, AD is characterized clinically by age-related anosmia (loss of smell), cognitive decline (decreased ability to think, reason, and perform complex mental tasks, ultimately leading to dementia), often difficult personality changes, and very commonly eventual verbal aphasia (inability to speak). Pathologically, AD is characterized by the presence of senile plaques (composed mostly of aggregated amyloid β-peptide (Aβ) and dystrophic neurites), neurofibrillary tangles (NFT, composed of aggregated hyperphosphorylated tau protein), and loss of synapses, and many scientists believe also loss of neurons [10]. Since both plaques and tangles are observable in brain of AD patients using PET scanning [11,12] and/or detection of both Aβ40 and Aβ42 and phosphorylated tau in CSF specimens [10], newer diagnostic criteria are more biochemical/pathological-centered and less on clinical symptoms, which can occur due to conditions other than AD.

Other biochemical/pathological alterations in AD brain, among others, include glucose dysmetabolism and Ca^{2+}dyshomeostasis, elevated oxidative stress-induced damage to proteins, lipids, and DNA/RNA, mitochondrial structural and functional abnormalities, neuronal structural changes, proteostasis network alterations, and differences in chaperone proteins [13]. Similar pathological changes, though to a lesser degree, are reported for brain in subjects with amnestic mild cognitive impairment (MCI), persons who have memory impairments but normal activities of daily living [14]. Preclinical AD is a relatively newly recognized stage of AD [15]. Preclinical AD persons have significant AD pathology but perform normally in tests of memory and cognition.

One of the most difficult aspects of AD is that pathology occurs well over two decades prior to symptoms [16]; therefore, means of identifying persons who will develop AD symptoms are desperately needed to initiate therapies as early as possible.

3. Proteomics

Proteomics is a term used to describe various methods to identify proteins in cells and cellular components [17,18]. Proteomics is an ideal technique to identify proteins with altered levels of post-translational modification. For example, identification of oxidatively modified proteins is the realm of redox proteomics [19–26], while phosphoproteomics, employed in the presence of phosphatase inhibitors, is used to identify differentially phosphorylated proteins in various matrices [4]. Although there are several methods for proteomics, the most often-used main components of proteomics involve protein and/or peptide separation, increase of the sensitivity of detection of post-translationally modified proteins, liquid-chromatography (LC) or nanospray tandem mass spectrometry (MS/MS) methods to sequence trypsin-generated peptides, and interrogation of databases to use these peptide sequences to identify proteins, since, of course, each protein has a unique amino acid sequence. Often, various approaches, such as western blotting, are used to validate the identity and trend in post-translational modification of proteomics-identified proteins. More details of redox proteomics or proteomics of other post-translational modifications can be found in various reviews or data papers [19–26].

3.1. Phosphoproteomics

The methods often used in phosphoproteomics were recently reviewed by Kempf and co-workers [27]. Likewise, others recent published methods of phosphoproteomics [28–30]. Immobilized metal affinity chromatography (IMAC) employs appropriate immobilized metal cations on various matrices that attract the negatively charged phosphate in chromatographic separations. Other metal methods, such as TiO_2-based methods are often employed. Kempf and colleagues combined these two methods in a process called sequential elution from IMAC (SIMAC), which has the advantage of separating mono-phosphorylated proteins from poly-phosphorylated proteins. Often both IMAC and SIMAC are combined with strong cation exchange (SCX) coupled with hydrophilic interaction liquid chromatography (HILIC). Modifications of this general approach using TiSH with the SCX and HILIC steps have proven useful for phosphoproteomics [27].

Isobaric tag for relative and absolute quantitation (iTRAQ) is a multiplexing method to simultaneously perform MS/MS proteomics on samples from up to 8 (newer versions suggest 16) different groups [21]. The method involves different stable isotopic composition of tags that bind to the N-terminal group and Lys and His side chains of proteins. Adroit use of ^{13}C, ^{15}N, ^{2}H isotopic substitution to amine-specific probes, coupled with an internal standard, permit this extreme multiplexing advantage for proteomics-mediated protein identifications based on sequencing tryptic peptides from multiple samples.

In addition to these above-referenced liquid chromatography-mass spectrometric (LC-MS) methods, multiplexed 2-dimensional gel electrophoretic (2DGE) methods for phosphoproteomics have been employed [31,32]. In this approach, samples are prepared in the presence of protein phosphatase inhibitors as well as the usual protease inhibitors routinely employed in proteomics studies. 2DGE separates proteins from cells or cell components based on isoelectric points in one dimension following isoelectric focusing, followed by traditional sodium dodecyl sulfate-gel electrophoresis in the second dimension. Individual proteins are stained by highly sensitive protein stains such as Sypro-ruby stain. After imaging these 2D gels to quantitate individual spots, a protein serine-, threonine-, and tyrosine-phosphate specific stain like Pro-Q diamond is applied. The spectral overlap of these two

stains is non-existent, so imaging the phosphorylated spots is easily accomplished by sophisticated imaging software. The pixel density of the phosphate stain for each spot is normalized to the protein pixel density of that spot, giving a specific phosphate level for a given protein spot. Statistical comparison of typically 6–10 individual normal vs. individual disease samples determines if there is increased, decreased, or no change in phosphoprotein levels between control and disease samples. The main disadvantage of this approach is the inability to know on the basis of the staining per se what are the amino acids on which the phosphate group(s) are bound. However, since proteins identified as different are subject to in-gel trypsin digestion followed by peptide clean up and LC or nanospray MS/MS, the sequence of the amino acids in the peptides examined are determined from which both the protein identification and the amino acids on which phosphate groups are bound can be determined.

Prior to the introduction of Orbitrap MS/MS methods, proteomics often were conducted using matrix-assisted laser-desorption ionization (MALDI) approaches [33–35]. In this approach, trypsin digests of proteins from 2-DGE of interest were placed in wells of a matrix composed of an aromatic acid, often α-cyano-4-hydroxycinnamic acid. Laser desorption of the protein and matrix led to a proton transfer to the peptides from the matrix in yet not understood manner, leading to ionization of the protein and its mass detection in the time of flight tube of the mass spectrometer. Interrogation of the tryptic peptide masses in appropriate protein databases led to a logarithmic MOWSE probability score of the identification of the protein. Scores beyond a specific cutoff were considered significant for the identification of the protein. This method is less often used presently due to the ability of modern mass spectrometers to sequence peptides directly by MS/MS methods.

In this review paper, we use the above considerations to discuss findings using phosphoproteomics in AD and MCI brain and in one case brain from individuals with preclinical AD, as well as in various in vitro and in vivo models of AD.

4. Phosphoproteomics in AD and model systems thereof

4.1. In vitro AD model systems

Using neuro-2a cells exposed to neurotoxic Aβ(25–35), stable isotope labeling of amino acids in cell cultures (SILAC)-based mass spectrometry was used to identify thousands of phosphoprotein labeling sites [36]. These researchers studied their system at 4 h and 24 h post exposure of this neurotoxic peptide. In addition, studies were conducted with this system also using a potentially protective drug (GFKP-19), described as a derivative of 2-pyrrolidine, which prevented many of these phosphosite changes in this AD model of neuro-2a cells. Of particular note and relevance to the known changes in AD, the researchers reported changes in phosphate labeling on nitric oxide synthase-1 (NOS1) and tau. NOS1, also called nNOS, is a Ca^{2+}-dependent enzyme located in the central nervous system, most often associated with the post-synaptic density-95 protein complex and is a key player in synaptic remodeling critical for learning and memory [37]. Since nitric oxide (NO), formed by NOS acting on arginine, is a free radical, it rapidly reacts with superoxide free radical via radical-radical recombination to form the non-radical peroxynitrite, $ONOO^-$. The latter in the presence of CO_2, leads to nitration of tyrosine at the 3-position [38]. As noted above, such sterically important binding to the 3-position of Tyr may lead to decreased activity of tyrosine kinases or tyrosine phosphatases, and also may contribute to the well-known elevated nitration of proteins in AD and MCI brain [34,39–42]. Yu and co-workers [36] showed that GFKP-19 decreased this phosphate-modulation of NOS1.

Tau phosphate binding sites determined by Yu et al. [36] (=#3 on list) were studied in deep detail. However, as discussed later by Yadav [43], not all tau phosphorylation sites are critical to tau function, so the significance of some of the phosphoproteomics results of Yu et al. [36] remain unknown. Additionally, a major criticism of this study is the experimental design itself. Namely, the authors used Aβ(25–35), which is neurotoxic, but is not known in AD brain, and, importantly, the neurotoxicity of this 11-mer peptide is entirely different than that of the 42-mer, Aβ(1–42) [44], which is highly relevant to neuronal loss, synaptic dysfunction, and insulin signaling decline in AD [45–47]. These concerns suggest that this study should be replicated with AD-relevant Aβ(1–42) oligomers.

Liu and Ni and co-workers [48] investigated the effects of selenate on phosphoproteins in N2aSW cells. These cells are derived from N2a neuroblastoma transformed cells that stably express human amyloid precursor protein (APP) with Swedish mutation that leads to familial AD. Others had shown sodium selenite treatment decreased γ-secretase activity leading to decreased Aβ production and protected rodents against cognitive loss in a chemically induced diabetes-related model of AD [49]. Moreover, selenate reportedly decreased tau phosphorylation by elevating activity of the protein phosphatase, PP2A, and led to protection against memory loss in some AD models [50,51].

The researchers examined both total phosphoproteins in selenate-treated N2aSW cells as well as phosphoproteins obtained employing a phosphoprotein enrichment approach. Two-dimensional gel electrophoresis followed by Pro-Q diamond staining, a stain specific for phosphoserine, phosphothreonine, and phosphotyrosine, to identify phosphoproteins. Sixty-five unique phosphoproteins were identified, with 60% of these proteins having increased phosphorylation and 40% having lower phosphorylation following selenate treatment compared to control cells. Functional analyses of the phosphoproteomics-identified proteins following selenate revealed proteins related to oxidative stress, proteasome function, glucose metabolism, cysteine and methionine metabolism, as well as other proteins involved in proteostasis, actin remodeling, and chaperone function. In each case, the elevation or decreased phosphorylation correlated with predicted improved function of the respective proteins. Importantly, these researchers also used biochemical analyses of this system to show decreased tau phosphorylation and decreased levels of Aβ, consistent with what promising therapeutics would do in AD patients. Indeed, seleno-L-methionine reportedly protects cells against Aβ or Fe^{2+}/H_2O_2-induced oxidative stress-mediated neurotoxicity [52], and prevented loss of cognition in a triple transgenic mouse model of AD [53], but caution is warranted against over-interpreting these findings, since Selenium-based agents in an AD-related clinical trial proved disappointing [54]. Still, the results of the Liu and Ni and coworkers [48] are intriguing, and more research on Se-containing compounds may be a potentially important approach to pursue in AD.

Padmanabhan and co-workers [55] studied neuroblastoma cells with the *APP* gene deleted (B103) or present (B103–695) using stable isotope labeling by amino acids in cell culture (SILAC) coupled with LC-MS/MS phosphoproteomics analyses with PolyMAC, a titanium-based nanopolymer for phosphopeptide enrichment. Unlabeled L-Arg and L-Lys were used for B103 cells, while $^{13}C_6$-L-Lys and $^{13}C_6$-$^{15}N_4$-Arg was used for B103–695 cells. Phosphoproteomics revealed not only differential phosphorylation of proteins in APP-expressing B102–695 cells, but specifically increased histone H4 phosphorylation at Ser47. In addition, when primary neurons were treated with oligomeric Aβ(1–42), histone H4 also was significantly phosphorylated at Ser47. The authors conclude that this specific histone, when phosphorylated, could play a role in altered transcription and cell cycle deregulation in AD. Other proteins also were detected by phosphoproteomics, including small heat shock proteins, similar to other studies noted above. In addition, phosphoproteomics identified cyclin-dependent kinase17 and cyclin-dependent kinase 18 as differentially phosphorylated in B103–695 cells compared to *APP*-null B103 cells, a result replicated by immunohistochemistry following Aβ(1–42) addition to primary neurons [55]. These researchers speculate that APP and Aβ(1–42) play roles in cell cycle dynamics involving Cdk17 and Cdk18 in AD.

In the same paper [55], these researchers also studied human

mutant presenilin/APP transgenic mice, albeit not using phosphoproteomics. Rather, western blotting validated the cellular findings that APP and Aβ contribute to Cdk17 and to some extent Cdk18 as well as histone H4 phosphorylation. Likewise, using control, MCI, and AD brain specimens provided by the University of California at Irvine Alzheimer's Disease Research Center, these researchers in the same paper demonstrated using western blots that these three proteins were abnormally phosphorylated in AD, with a trend toward increased phosphorylation in MCI specimens both relative to control brain. The mean postmortem intervals (PMIs) of controls, MCI, and AD specimens were 4.3, 4.9, 4.7 h, only slightly higher than the desired 3 h or fewer for PMI (see further discussion below).

4.2. In vivo AD and aging model systems

Colton and colleagues investigated the effects of aging on differential protein expression in an AD mouse model in which the gene for *inducible nitric oxide synthase* (i-NOS) was knocked out [56]. The researchers employed brains isolated as a function of age from a novel mouse model of AD: the APPSwDI/NOS2$^{-/-}$ (CVD-AD) mouse. Phosphorylated tryptic peptides enriched for phosphate by an automated LC TiO_2 system (IMAC) followed by LC-MS/MS, identified > 1100 phosphopeptides. Database searching let to the identification of phosphorylated brain proteins as a function of age of this AD mouse model. Phosphorylation of the protein, connexin 43, which had not been previously known in AD, was observed at nearly 11 months of age, which the authors suggest could reveal dysregulation of gap junctions and activation of astrocytes. Moreover, differential phosphorylated proteins as a function of age were identified as being derived from mitochondria or involved in synaptic transmission, vesicle trafficking, and innate immune pathways. The authors conclude that the CVN-AD mouse model of AD provided novel insights into age-dependent alterations to brain proteins during disease progression.

Kempf and co-workers investigated different brain regions of the double human mutant $APP_{SWE}/PS1_{\Delta E9}$ AD mouse model using phosphoproteomics along with other proteomics modalities [57,58]. Changes in phosphorylation of proteins related to synaptic functions secondary to dendritic spine density and morphology and cytoskeletal dynamics were observed at 12 months in male mice. The main foci of these studies were the aberrant phosphorylation of stathmin1, which regulates microtubule dynamics, and tau, which stabilizes microtubules and as mentioned when hyperphosphorylated leads to NFTs and neurodegeneration. The authors suggest that changes in dendritic processing and spine density are consequently affected, which could be important in AD in loss of learning via long-term potentiation.

Wang and colleagues utilized triple isotope demethylation labeling coupled with SCX and hydrophobic HPLC separations to enrich phosphopeptides in order to determine age-dependent alterations in the hippocampal proteome of the TgCRND8 mouse model of AD [59]. These investigators were able to distinguish phosphoproteome changes in the transition from presymptomatic to symptomatic states of this mouse. Thirty-six phosphoproteins associated with synaptic function and cytoskeletal structure were suggested to be key to this transition to the symptomatic state in this mouse model of AD, and were suggested to comprise a set of promising therapeutic targets for AD.

Tagawa et al. examined four types of mouse models of AD: APPTg2576, PS1-DE9, PS2-M1, and the 5 X FAD mouse for changes in the brain phosphoproteome [60]. A human mutant tau mouse (P301S) also was examined. Data obtained were subjected to analysis using a supercomputer and methods of systems biology. Seventeen phosphoproteins involved in glucose metabolism, axonal and spine morphology, synaptic vesicle function, and cytoskeletal structure were identified as different in these mouse models compared to those in WT mice brains. The authors' network analyses revealed that 12 of these 17 phosphoproteins were related to phosphorylation of myristoylated alanine-rich C-kinase substrate (MARCKS) by kinases that become hyper-activated and lead to synaptic pathology prior to deposition of Aβ, consistent with the notion that these processes may be critical to early events in neurodegeneration.

Chronic low-dose rate ionizing radiation was used to investigate the effects of such radiation on the hippocampal phosphoproteome of the ApoE$^{-/-}$ knock-out mouse on a C57B1/6 background [61], an AD-related mouse model [62,63]. Apolipoprotein E, involved in cholesterol transport and Aβ removal from brain, among other functions, has three different alleles, E2, E3, and E4. Persons who inherit *ApoE4/4* have about a 70% chance of developing late-onset AD, while persons with *ApoE2/2* have only a marginal chance of developing AD [64]. So, while *ApoE4* does not cause AD, this gene does play a role in risk of developing AD. These researchers exposed these mice to either 1 mGy/day or 20 mGy/day over 300 days giving cumulative doses of 0.3 Gy or 6 Gy, respectively. As noted above, the authors improved methods of identifying phosphorylated peptides, and using these methods phosphorylated proteins associated with synaptic plasticity, Ca^{2+}-dependent signaling, and brain glucose metabolism were found to be altered following ionizing radiation. Specifically, CREB signaling inhibition was found at both radiation doses, while phosphorylated proteins involved in Rac1-Cofilin signaling were activated at the lower radiation dose only. Biochemical correlates of low-dose radiation included decreased number of activated microglia, lower levels of lipid peroxidation, and lower levels of the pro-inflammatory cytokine TNFα. The authors conclude that low-dose ionizing radiation leads to phosphorylated proteins whose associated functions overlap with known changes in AD brain, and they speculate that ionizing radiation may contribute to elevated risk of developing AD [61].

In contrast to the detrimental effects of altered phosphorylation levels in these AD in vitro and in vivo models mentioned above, phosphoproteomics investigations of brain proteins as a function of animal age pointed to potential mechanisms for increased survival associated with elevated health span and life span in the naked mole rat, a very long-lived rodent [65–67]. The naked mole rat lives up to 32 years in marked contrast to the typically 3-year maximum lifespan of mice and rats, and unlike the latter, the naked mole rat appears never to develop cancer [68]. Better understanding of the differential phosphorylation of brain proteins in these long-lived rodents may provide new insights into increased health span that conceivably may one day be translatable to humans.

4.3. Phosphoproteomics investigations in AD and its earlier stages

As noted in two recent reviews of phosphoproteomics [4,27] and mentioned above, there are relatively few papers on phosphoproteomics in AD brain. Nevertheless, this is a key area to exploit since new insights of high relevance to this disorder are possible as will be described presently.

The work of Coulton and colleagues on the CVN-AD mouse was discussed above [56]. In the same paper, these researchers used phosphoproteomics to investigate the frontal cortex from control and AD specimens provided by the Emory University Brain Bank. Classification of individual cases was based on phosphopeptide signal intensities and demonstrated 253 phosphopeptides were present in abundance of either at least 1.75-fold higher or at least 1.75-fold lower than the respective controls. These phosphopeptides sorted into three groups, two of which had higher levels of phosphopeptides, and the third lower, in AD frontal cortex relative to controls. Proteins related to the cytoskeleton were in each group. The two groups with higher phosphopeptide levels were functionally over-represented by proteins associated with the plasma membrane, glycoproteins, cytoskeletal binding, the synapse, and those of alternative splicing. The phosphopeptide group was rich in proteins associated with the endoplasmic reticulum. Tau was highly represented in the results. Interestingly, small heat shock protein phosphorylation was correlated with a group of kinases. Such HSP phosphorylation regulates their chaperone activity, which is decidedly

dysfunctional in AD and conceivably could be related to the findings in this paper. The results validated the use of the CVN-AD mouse model (described above) as a good way to investigate aspects of AD.

A concern associated with the specimens provided by Emory University to these researchers is the large postmortem interval (PMI) involved. For controls, the mean PMI was about 7 h, while for AD specimens, the mean PMI was about 6 h. It is clear that many changes can occur in a brain that is not harvested for this long a time following death, among which could reasonably be expected to be the status of its protein phosphorylation. Repetition of these studies is warranted using specimens obtained at PMIs of < 3 h, arguably the fastest PMI reasonable.

A highly similar study as that of Coulton and colleagues [56] on human AD specimens was reported in 2015 [69]. Seyfried and colleagues used IMAC coupled to LC-MS/MS to identify 253 phosphopeptides with elevated or decreased levels of phosphate in 8 AD specimens compared to 8 control specimens. Similar conclusions were obtained as those of Coulton and co-workers, including the groups of proteins identified from the elevated and decreased phosphopeptides, the relationship of these findings to small heat shock proteins, etc. Examination of the PMIs of this Seyfried and co-workers study shows essentially identical values as those of Coulton and co-workers in their study published in 2013. It is this author's opinion that these samples, both from Emory University, were likely identical or highly similar in the two studies. What is surprising is that the Seyfried and co-workers study did not cite the Coulton and co-workers study published two years earlier. That the two independent laboratories reached the same conclusions, gives confidence to the results obtained using the samples provided. However, the same criticism of the Coulton and co-workers study applies to the Seyfried and co-workers study: the PMIs are quite long and quite likely led to significant biochemical changes from time of death to time of brain isolation, including phosphorylation levels, thereby not representing the actual brain at time of death with high fidelity. A similar caution applies to the Seyfried study as the Coulton study: The results likely should be considered tentative until replicated with specimens from AD and control brains obtained at a PMI of 3 h or fewer.

Neurofibrillary tangles are known to be composed of aggregates of hyperphosphorylated tau protein in AD brain. Loss of tau from microtubules has highly detrimental effects on neurons related to loss of the vesicle and mitochondrial transport from the cell body to the presynaptic terminus and loss of part of the cytoskeletal network of neurons in this disorder. Presynaptic membranes would be starved for energy as a consequence of ATP loss, and changes in the cell potential would occur, allowing huge amounts of detrimental Ca^{2+} to enter neurons via voltage-gated Ca^{2+} channels. However, although another cytoskeletal network set of proteins, the neurofilament proteins, were known also to be part of NFTs, though the identity of these neurofilament proteins, i.e., heavy-, medium-, or light-weight neurofilament proteins, was unknown. Pant and colleagues [70] used IMAC TiO_2-associated phosphoproteomics to definitively identify all three neurofilament phosphorylated neurofilament proteins to be associated with NFT. In addition, phosphorylated vimentin, an intermediate filament protein, also was identified as present in NFT. These results, if validated by additional studies, extend our knowledge of the composition of NFT in AD brain. Two criticisms of this study are: 1) Brains from only two AD and two control subjects were studied, which leads the current author to suggest these findings, though elegantly obtained, should be considered preliminary until additional specimens are examined; and 2) no indication of PMI was provided, and as noted above, a long PMI could materially affect phosphorylation status.

Steen and co-workers developed a new method to map the phosphate landscape on the tau protein in AD brain [71]. The method, full length expressed stable isotope-labeled tau (FLEXITau), was used for tau isolated from AD brain to show the entire phosphorylation location on this protein, from which the authors propose that new therapeutic targets conceivably might arise. While this method is certainly novel and potentially useful in AD research, the concern remains that not all phosphorylated sites on tau are pathogenic and this method appears not to distinguish which sites are key to tau's role in AD and which are more benign. Future studies undoubtedly will clarify this situation.

In arguably the first widespread phosphoprotein analysis of AD brain, Peng and colleagues enriched tryptic phosphopeptides from AD brain by forming precipitates of calcium phosphate after addition of Ca^{2+} and determined identity of phosphoproteins using LC-MS/MS sequencing of the phosphopeptides [29]. Changes in phosphorylation status of proteins associated with the cytoskeletal network (including neurofilament proteins, microtubule associated proteins, spectrin and actin, among others), synaptic proteins (including MARCKS), and a chaperone protein (heat shock protein 90) were reported. The major criticism of this work is the acknowledgement by the authors that the PMI of specimens obtained was up to 20 h. As noted above, this extraordinarily long PMI is not a good representation of the state of the AD brain, and consequently, to ensure the utility of this clever approach to phosphoproteomics in AD, it would need to be repeated on specimens obtained in very short PMIs (typically < 3 h).

The same group refined their phosphoproteomics methods and tested the method using a much shorter PMI [30]. In particular, TiO_2 in the presence of KH_2PO_4 resulted in a substantial increase in phosphopeptides identified. These researchers tested this refinement in one well-characterized AD case, obtained from Banner Sun Health Research Institute with a PMI of < 3 h. As in their earlier study, synaptic proteins (particularly MARCKS), cytoskeletal proteins, and a chaperone protein were identified as abnormally phosphorylated. However, as noted, this was with one AD case [#62 PERLUIGI REV], which requires validation with greater number of individual specimens.

The study by Tagawa et al. on different mouse models of AD was discussed above [60]. Also reported in this paper were phosphoproteomics results in preclinical AD brain. The authors found that MARCKS, which affects actin polymerization that is key to learning and memory following synaptic remodeling, was differentially phosphorylated. MARCKS phosphorylation also is key to dendritic spine density, the latter a key correlate of cognitive performance. As such, there is significant overlap of the results from the various transgenic mice studied by this group and the human samples, given confidence in the results and validation of the transgenic mice studies. Tempering this assessment however, this paper did not contain information on PMI of specimens from human subjects, so in this author's opinion, this highly comprehensive study should be taken as preliminary until either PMI information becomes available or until the study is repeated with specimens obtained in a short PMI (< 3 h).

Zahid and colleagues used a multiplexed approach to phosphoproteomics analyses of the cortex and substantia nigra regions of AD brain [72]. Two-dimensional gel electrophoresis of samples was obtained, followed by protein staining and Pro-Q diamond staining as described above. The authors found that about two-thirds of the altered phosphorylation of proteins were involved in: glucose metabolism (32% of total number of phosphoproteins identified); signal transduction (16%); cytoskeletal network (10%); and apoptosis (6%). As with several of the prior studies of phosphoproteomics, it is the opinion of this current author that these results have to be viewed cautiously, since the PMI reported was approximately 24 h. After such a long PMI, the reality of the brain at that time is certainly not reflective of the brain at the time of death or shortly thereafter.

The Butterfield laboratory published two phosphoproteomics studies in AD brain and its earlier stages [31,32]. In the first, the hippocampus was examined from AD and normal control subjects whose mean PMI was < 3 h [31]. Seventeen phosphoproteins of differential phosphorylation levels were identified, among which several glycolytic enzymes were known to be dysfunctional. Also, other glucose metabolism-related mitochondrial enzymes were identified, as were phosphoproteins involved in cell signaling.

Table 1
Compared to the respective control, fold-change in phosphorylation[a] and functional increase (up arrow) or decrease (down arrow) of inferior parietal lobule proteins in preclinical AD (PCAD), amnestic mild cognitive impairment (MCI), or late-stage Alzheimer disease (AD). Functional classes are also shown[b]. Based on results from [32].

Phosphoprotein	Functional class	PCAD		MCI		AD	
		Fold-change	Function change	Fold-change	Function change	Fold-change	Function change
Heat shock protein 70	Ch	8.9	↑				
Gelsolin	Ca, Sy	0.28	↓	0.38	↓	0.18	↓
Regucalcin	Ca, Ox	0.017	↓	0.026	↓	0.11	↓
Lactate dehydrogenase	M	28.7	↓			34.8	↓
Voltage-gated anion channel protein-1	Mi	0.23	↓			0.15	↓
Septin-2	Si, Sy	2.9	↑				
Flavin reductase (biliverdin reductase-B)	Ox,	8.9	↑				
2′,3′-Cyclic nucleotide-3-phosphodi-esterase	St, Si	0.008	↑				
Citrate synthase	M			3.9	↑		
EF-hand domain-containing protein D1	Ca, Ox, Mi			0.07	↓	0.08	↓
Transitional ER ATPase (valosin-containing protein)	Ps			0.057	↓	0.26	↓
Voltage-gated anion channel protein-2	Mi					0.083	↓
Dihydropyrimidinase-related protein-2 (collapsin response mediated protein-2)	Sy, Cy					3.5	↓
Guanine nucleotide-binding protein G(0) subunit α	Si					6.1	??
Cu/Zn superoxide dismutase	Ox					0.01	↓
Peroxiredoxin-1	Ox					0.049	↑
Carbonyl reductase-1	Ox					0.39	↑
Stathmin	Cy					0.045	↑
Syntaxin-binding protein-1	Sy					0.18	↓

[a] Fold-change = 1 means no change from control. Numbers > 1 indicate increased protein phosphorylation, while numbers < 1 indicate decreased phosphorylation, respectively, from their respective controls.

[b] Functional classes: M: metabolism; Ca: calcium-related function; Sy: synaptic function; Ox: oxidative stress-related; Mi: mitochondrial; Si: signaling; St: structure; Cy: cytoskeleton; Ch: chaperone; Ps: proteostasis network protein; ??: effect of phosphorylation level on activity unknown.

In the second paper from our laboratory [32], a different brain region, the inferior parietal lobule, a brain region rich in pathology in AD, also obtained with a mean PMI of < 3 h, was investigated throughout the spectrum of AD progression, i.e., preclinical AD, amnestic MCI, and late-stage AD. The same multiplexed 2DGE-based approach described above was used in this, the first study to determine the changed inferior parietal phosphoproteome throughout the progression of AD. Our main hypothesis of the study was that altered phosphorylated proteins that are identified in each stage of AD are critically involved in the pathogenesis and progression of this dementing disorder. Table 1 shows the proteins with altered phosphorylation in each stage of this disease, their functional classes, whether the change in phosphorylation causes an increase or decrease in function.

In addition to a number of proteins that had significantly different levels of phosphorylation from their respective controls in two stages of the progression of AD and therefore might have significance in the progression of AD, two key proteins, gelsolin and regucalcin, had significantly decreased phosphorylation in all three stages of AD progression compared to the respective aged-matched controls. Gelsolin catalyzes the severing of F-actin polymers in processes related to synaptic remodeling, particularly associated with growth cones needed to form new synaptic connections and in processes related to learning and memory [73,74]. A gelsolin C-terminal cleavage product, following action of caspase-3 during apoptosis, is both increased in AD brain and correlates with AD severity [75]. Moreover, gelsolin binds to Aβ, sequestering this neurotoxic and oxidative stress-related peptide, decreasing the detrimental properties of the latter, including Aβ-induced apoptosis and neurotoxicity [76,77]. The consequent decreased activity of gelsolin due to decreased phosphorylation at each stage of AD progression is consistent with decreased sequestration of Aβ oligomers and consequent increased neurotoxicity. See discussion above on MARCKS that deals with actin polymerization with respect to its altered phosphorylation in AD.

Regucalcin (also called human senescence marker protein 30, SMP30) is a Ca^{2+}-dependent protein that is involved in regulation of membrane Ca^{2+} pumps [78], and decreased regucalcin reportedly causes dysregulation of Ca^{2+}-dependent kinases and phosphatases and increases oxidative stress [79,80]. The role of phosphorylation on enzymatic activity of regucalcin is presently unknown, but the results show that in all stages of AD phosphorylation of this protein barely is detectable compared to the respective controls. We speculate that this significantly decreased phosphorylation leads to loss of function.

The findings and significance for AD of these two papers are consistent with new studies involving patients in the dominantly inherited Alzheimer disease network (DIAN), an ongoing study involving thousands of persons from three continents known to have mutated genes that lead to familial AD [81]. From as long as 22 years prior to and 3 years after the onset of clinical symptoms (25 years), these persons underwent regular scans using various imaging modalities (PET scanning for Aβ deposition; PET scanning for glucose metabolism; MRI to observe cortical and hippocampal thickness as a surrogate marker for neuronal loss) to determine what is the order of neuropathological changes in AD. Well before the onset of clinical symptoms, the DIAN study showed aggregation and deposition of Aβ, presumably also including Aβ oligomers, occurred first followed by decreased glucose metabolism, and finally by decreased hippocampal and cortical thickness [81]. Oligomeric Aβ is known to be associated with oxidative stress, including oxidative modification of glycolytic and TCA cycle enzymes and ATP synthase (leading to less ATP formation) [38–42,82–84], which would lead to loss of cell potential and consequent opening of Ca^{2+} channels and accumulation of intracellular Ca^{2+}. This intracellular Ca^{2+} cannot be pumped out efficiently because both regucalcin is hypophosphorylated and the Ca^{2+}-ATPase and Na/Ca^{2+} exchanger are oxidized by Aβ [85,87]. Both necrosis and apoptosis would occur, leading to neuronal death and consequent loss of cognition.

5. Conclusions and future studies using phosphoproteomics in AD

As noted early in this paper, there are relatively few published

papers on phosphoproteomics studies in AD and models thereof. Regrettably, a significant proportion of those involving human brains report results on specimens obtained with an extraordinarily long PMI, up to 24 h after death. These studies with long PMIs may provide important information, but one has to be concerned that the brain at that long a PMI would not have high fidelity with the living brain, and, therefore, would not provide reliable or accurate information germane to what is the true situation regarding phosphorylation status. Investigators planning to pursue phosphoproteomics of human brain in the future are strongly urged to use brain specimens obtained at a PMI < 3 h from well-characterized AD, MCI, and PCAD subjects and their respective aged-matched controls. Anything else poses serious risks of artefactual results.

The methods of phosphoproteomics have improved with time, to now provide results with high precision and accuracy. Likely to be expanded in the future from present approaches [27], the ability to use proteomics to identify proteins with more than one post-translational modification would be especially welcomed [86]. This is because many of the phosphoproteomics-identified brain proteins in AD and its earlier stages also are oxidatively modified [20,21,31–35,39–42,45,82–84,87], glutathionylated [88,89], or otherwise modified [90]. It is widely accepted that brain pathology occurs around two decades prior to the onset of symptoms [16,81], necessitating easily obtained, AD-specific biomarkers to be identified that could be used to determine with high confidence whether a person is or is not of high risk of developing AD. Neuronally derived, plasma-resident extracellular vesicles, for example, could be subjected to phosphoproteomics to conceivably identify such biomarkers.

With attention to the biological and pathological states of specimens obtained at autopsy, the future is bright for phosphoproteomics in the study of brain from AD and other neurodegenerative disorders. It is highly possible that new therapeutic targets to slow or stop AD will emerge from such studies.

Transparency document

The Transparency document associated with this article can be found, in online version.

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