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Uncovering neurodegenerative protein modifications via proteomic profiling

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Abstract

Degenerative protein modifications (DPMs) are caused by non-enzymatic chemical reactions that induce changes in protein structure and function which promote disease initiation, pathological progression, and also natural ageing. These undesirable DPMs include oxidation, carbonylation, carbamylation, glycation, deamidation and racemization, which impart deleterious structural and functional changes on extracellular matrix proteins and long-lived cell types such as cardiomyocytes and neurons, leading to impaired overall organ function. Despite the obvious clinical importance of understanding DPM biology, the molecular mechanisms that mediate these modifications remain poorly understood largely due to the technical challenges associated with their study. However, recent advances in mass spectrometry-based proteomics technologies now permit global quantitative proteomic profiling of cell lines, animal models and clinical samples from a variety of different patient types. These new methods have not only uncovered changes in global protein expression levels, but have also identified specific modifications of particular amino acid residues in protein backbones that are associated with disease progression. The non-enzymatic induction of DPMs as revealed by proteomic profiling can help us to better understand the underlying molecular pathology of protein dysfunction in human diseases and natural aging. This chapter will discuss recent progress in understanding how proteomic profiling of patient samples derived from the central nervous system can elucidate the DPM biology of human neurodegenerative diseases.

Keywords: Protein dysfunctions; Neurodegeneration; Dementia; Mass spectrometry; Posttranslational modifications.

Abbreviations: CNS, central nervous system; CR, congo red; AD, Alzheimer's Disease; PD, Parkinson's disease; HD, Huntington's disease; ALS, amyotrophic lateral sclerosis; MAPT, microtubule associated protein Tau; PTMDPs, posttranslational modification derived products; ROS, reactive oxygen species; UBS, ubiquitin proteasome system; AGE, associated glycation end-products; iTRAQ, isobaric Tag for Relative and Absolute Quantitation; VaD, Vascular Dementia; PTN, protein tyrosine nitration; NO, nitric oxide.

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Introduction

The study of protein dysfunction in diseases of the central nervous system (CNS) dates back over 150 years, but progress in understanding the pathology of neurodegenerative diseases has been limited by the lack of suitable methodologies for unravelling their molecular basis. In 1854, the German physician Rudolph Virchow used best histochemical methods available at the time to identify wax-like plaques in brain tissues from autopsied individuals. Those unfamiliar features acquired bluish tones when stained with iodine and sulfuric acid, leading Virchow to conclude that these plaques were composed of 'starch'. Accordingly, he dubbed these plaques 'amyloid', which is derived from the Latin and Greek terms for starch (see Cohen 1967 for a comprehensive history of amyloidosis (Cohen, 1967)). Five years later, Friedrich and Kekule suggested that brain amyloid plaques were in fact composed of proteins rather than carbohydrates, and in so doing they established for the first time the presence of protein dysfunctions in the brain (Sipe and Cohen, 2000). Early in 20th century, the generalized use of congo red (CR) stain allowed the description of the fibril composition of amyloid plaques, as revealed by the contrasting red and the green tones exhibited by stained samples when visualized under polarized optics (Cohen and Calkins, 1959).

From the first discovery of brain amyloidosis in 1854, the presence of protein dysfunctions have been described in almost all neurodegenerative disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS). The amyloid- β peptide and the microtubule-associated protein tau (MAPT) are the key constituents of brain plaques and tangles in AD patients, α -synuclein protein is the main component of brain depositions in PD, huntingtin oligomerization is characteristic of HD brain tissues, and the proteins TDP-43 and SOD1 are typically misfolded in ALS. The tendency of dysfunctional proteins to aggregate and form depositions in neurodegenerative diseases, and the subsequent toxic effects exerted by these fibrillations on brain cell function remain poorly understood. Nonetheless, it is remarkable how the presence and the quantity of plaques in the brain does not correlate with decline in cognitive functions during dementia (Terry et al., 1991).

The initial focus of investigations into the biology of brain amyloidosis was centered on protein oligomers themselves as putative key mediators of the neurodegenerative process. Indeed, this initial hypothesis that protein oligomers initiate proteinopathies has for many years shaped the overall direction of research in neurodegenerative pathologies. However, recent data have indicated that a diverse range of soluble proteins can become dysfunctional and exert toxic effects in brain tissues that surpass those of oligomerized proteins. In spite of this development, there are still only a few reports that have successfully documented the extent of protein dysfunction, modified composition, and altered conformation that occur in neurodegenerative disease. However, as discussed in this review, the most recent technical advances in mass spectrometry-based proteomics have now provided us with the necessary tools to uncover the molecular basis of protein dysfunction in diseases of neurodegeneration.

Non-enzymatic protein modifications and protein dysfunctions

Posttranslational modifications (PTMs) regulate protein structure and functions (Mann and Jensen, 2003; Merkley et al., 2014). In healthy cells and tissues, PTMs induced by

chemical/enzymatic modification of protein side chains represents a common mechanism of altering protein function, whereas PTMs generated by non-enzymatic or spontaneous chemical processes are associated with the initiation and progression of disease processes (including inflammation, oxidative stress, proteostatic imbalance, and proteinopathy (van Kasteren et al., 2007; Victorino et al., 2014; Halim et al., 2015)). The protein chemical modifications that take place *in vivo* can occur either spontaneously (e.g. deamidation and racemization), via non-enzymatic chemical reactions (e.g. oxidation and carbamylation), or due to enzymatic activity (Gillery and Jaisson, 2014; Mommen et al., 2014). The non-enzymatic and spontaneous modification of protein side chains are usually associated with loss of function, hence these have been termed degenerative protein modifications (DPMs) in the current review. Protein DPMs are considered to be a natural consequence of molecular aging, and all known proteins are susceptible to DPMs during their lifespan. However, the presence of DPMs in some long-lived host proteins has been reported as associated with pathogenic conditions and diseases (Gillery and Jaisson, 2013; 2014). The DPMs already known to be associated with degenerative diseases and human aging include oxidation, racemization, isomerization, deamidation, nitration, carbonylation, carbamylation and glycation. Unfortunately, most of these DPMs are irreversible chemical modifications, hence the defective products of these modifications tend to accumulate in the tissues and can eventually impair overall organ function (Soskic et al., 2008).

DPMs typically exert detrimental effects on the affected proteins. For example, protein oxidation causes severe structural damage due to peptide backbone cleavage, which can lead to the pathological accumulation of misfolded proteins (Berlett and Stadtman, 1997). Oxidation has also been implicated in mediating the pathological effects of smoking, chronic alcoholism, harmful radiation and ischemia, since all of these environmental stressors promote the massive production of reactive oxygen species (ROS) in host cells (Schuessler and Schilling, 1984). Similarly, protein racemization which changes L-amino acid to D-amino acid occurs during peptide backbone hydrolysis or deamidation, hence this DPM mainly promotes protein degradation (Powell et al., 1992; Ritz et al., 1996). Racemization can also result from the acquisition of a succinimide ring that forms during deamidation, leading to changes in protein conformation prior to degradation (Clarke, 1987; Lowenson and Clarke, 1988; Fabian et al., 1994; Orpiszewski and Benson, 1999; Ritz-Timme and Collins, 2002). It has been proposed that racemization can promote protein oligomerization and cellular toxicity when mechanisms of protein quality control are disrupted e.g. suppression of the ubiquitin-proteasome protein degradation pathway. Formation of succinimide rings can occur due to the rapid deamidation of asparaginy or glutaminy residues, but may also arise as a consequence of the slower processes of aspartyl or glutamyl isomerization. In these cases, not only do the resultant protein DPMs promote racemization, but they also lengthen the protein backbone via the addition of atypical amino acid structures known as iso-aspartic acid and iso-glutamic acid (Ritz-Timme and Collins, 2002). Oxidative damage to proteins arising from transcriptional or translational errors has also been proposed as an important precursor of asparagine and glutamine deamidation *in vivo* (Dukan et al., 2000). Deamidation of an asparagine or glutamine residue changes the chemical properties of protein side chains (amide to carboxyl) and in some cases modifies the orientation relative to the rest of the polypeptide chain (Hipkiss, 2006). Accordingly, spontaneous deamidation has been identified as a major source of protein instability and damage (Gupta and Srivastava, 2004; Adav et al., 2014). In the same light, it has been proposed that cellular production of

ROS promotes the rearrangement of protein structure and consequently induces 'blooming' of isomerized residues (D'Angelo et al., 2005). The effects of isomerization on protein structure and function are thought to be largely similar to those of racemization and deamidation. Indeed, both spontaneous deamidation and isomerization can promote protein misfolding and impair basic molecular functions, while also being implicated in the acquisition of toxic characteristics (Desrosiers and Fanéus, 2011).

Among common DPMs, nitration has perhaps been the most clearly shown to induce protein dysfunction. The impact of protein nitration on the function of ATP-synthase has been studied extensively, and it is now clear that accumulation of nitration products can disrupt the ability of this enzyme to synthesize adenosine triphosphate (ATP) (Abdelmegeed et al., 2013). It has also been demonstrated that accumulation of protein nitrations can delay protein degradation via the ubiquitin proteasome system (UPS) (Souza et al., 2000), resulting in defective protein aggregation in the affected cells and tissues.

The detrimental effects of oxidative stress on protein function are manifold due to the variety of DPM products this can generate, including oxidized, cleaved and carbonylated proteins that each exhibit impaired function. For example, the ATPase *SERCA2A* displays reduced ability to hydrolyze ATP following carbonylation of its component lysine or arginine residues (Shao et al., 2011). Similarly, another common DPM known as carbamylation is thought to result from ROS production in host cells, leading to uremia toxicity in end-stage renal disease. All carbamylated proteins identified in previous studies of uremia were marked by total loss of normal biological activity (Erill et al., 1980; Fluckiger et al., 1981; Wang et al., 2007). Accordingly, protein carbamylation has now also been implicated in inflammation, atherogenesis and smoking-induced chronic diseases (Wang et al., 2007).

In environments that are rich in reduced sugars, proteins may undergo amino acid glycation or form advanced glycation end-products (AGEs) (Brownlee et al., 1988; Ruderman et al., 1992; Bucala et al., 1993). Proteins rich in lysine residues are particularly prone to the accumulation of glycations and AGEs (Yan et al., 1994a), leading to differential modulation of protein function depending on the intra/extracellular location of the affected molecule. AGE-bearing proteins located inside cells tend to oligomerize and represent a source of ROS that can damage multiple other proteins and cellular organelles. Similarly, extracellular matrix proteins that accumulate AGEs can acquire the ability to interact with several important receptors, e.g. the receptor for ages (RAGE); a specific type I cell surface receptor or the macrophage scavenger receptor A (SC-A) (Ott et al., 2014). Agonistic interactions of AGEs to their binding receptors activate many cellular pathways as the NF κ B pathway, microglia pro-inflammatory pathways, etc. inducing oxidative damage to surrounding cells (Sakurai et al., 1990; Yan et al., 1994b).

Analysis and characterization of DPMs by mass spectrometry-based proteomics

The central dogma of molecular biology states that one gene encodes for a single protein with a defined function, but recent advances achieved in the field of proteomics have challenged this concept by revealing that myriad PTMs can diversify the end products of individual genes (Cain et al., 2014). In 1959, Fischer et al. demonstrated for the first time

that certain amino acids in a protein sequence can be modified by the addition of a phosphate group (phosphorylation) (Fischer et al., 1959). This seminal discovery was followed 4 years later by the first report that histones could feature acetylations, that would later be determined to exert potent effects on gene expression levels. At the time of these discoveries, standard molecular techniques allowed only the direct analysis of a single protein modification in a purified sample, and detailed analysis of varied PTMs across the whole proteome would remain unreachable until many decades later. Indeed, even after the development of two-dimensional gel electrophoresis, which finally enabled the large-scale study of protein PTMs in human clinical samples, the limited sensitivity of this technique was unable to detect the small molecular weight changes that denote specific DPMs. As a result, the detailed characterization of DPMs in complex mixtures required the evolution of mass spectrometry, which offered substantially increased sensitivity and dynamic ranges of detection than were previously available. Characterization of protein PTMs and/or DPMs by mass spectrometry can be performed using either top-down or bottom-up proteomic methods (Moradian et al., 2014). Top-down proteomics aims to directly analyze intact proteins without prior digestion during sample preparation. This approach can detect any type of protein modification arising from chemical modification of the constituent amino acids by measuring mass changes in the precursor ion and resultant fragments during analysis. Although top-down proteomics is perhaps the most suitable approach for the characterization of PTMs/DPMs in complex patient samples, this technique is limited by low protein ionization efficiency as well as the inability of current mass spectrometry technology to efficiently fragment and analyze large protein ions (Dang et al., 2014). At the present time, bottom-up proteomics is a more mature technology that enables proteomic analysis of PTMs/DPMs profiles at the peptide level (Tran et al., 2011; Moradian et al., 2014). This more practical method of DPM analysis uses peptide separation by liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) to analyze trypsin digested peptides. The major limitations of the bottom-up approach are the requirement to perform trypsin digestion prior to analysis, difficulties achieving 100% sequence coverage of proteins (due to loss of improperly digested peptides), and the generation of peptides that are too short/long for robust characterization. In addition, some hydrophilic peptides that exhibit potentially important PTMs/DPMs are not retained in the reversed-phase C₁₈ column required to employ the bottom-up approach (Olsen and Mann, 2013). In addition, the identification of relatively low abundance DPM-bearing peptides in complex patient samples is somewhat limited by the dynamic range of current mass spectrometry instruments. However, various enrichment and chromatographic methods have been developed that enable the enrichment and separation of peptides with specific PTMs/DPMs on a proteome-wide scale (Olsen et al., 2004).

The characterization of DPMs in complex biological samples is usually restricted by the presence of artifactual DPMs that are generated by the various buffers used in sample preparation. For example, bottom-up proteomic analysis commonly employs a urea buffer which can produce carbamylated protein/peptide artifacts, and optimal trypsin activity at pH 8 can induce experimental protein deamidation. To avoid generating these artifactual DPMs during sample preparation, our group developed a new method of sample processing which substantially improves the accuracy with which protein deamidation can be identified in biomedical samples (Hao and Sze, 2014). This method uses ammonium acetate buffer at pH 6 throughout sample preparation, which maintains sufficient trypsin activity

for protein digestion while also restricting the kinetics of asparagine deamidation, thereby minimizing the generation of artifactual deamidation products (Hao et al., 2011; Hao and Sze, 2014).

Figure 1 Schematic diagram showing the bottom-up approach (upper) and top-down approach (lower) to protein characterization and proteome analysis. [Figure adapted from Science, 2006, 314, 65, 2006]

Study of the Brain Proteome by Quantitative Proteomics

The development of mass spectrometry-based quantitative proteomics, using either label-free approaches or stable isotope labeling (including tandem mass tag [TMT] and isobaric tag for relative and absolute quantitation [iTRAQ]), presented new opportunities to analyze and characterize the whole brain proteome in both health and disease. Our group pioneered the application of these techniques to the study of changes in the human brain proteome in both ischemic stroke (Datta et al., 2010; Datta et al., 2011; Datta et al., 2013) and vascular dementia (VaD) (Adav et al., 2014; Datta et al., 2014b; Gallart-Palau et al., 2015). For the VaD study, we pooled post-mortem brain tissues from 10 healthy subjects and 10 VaD patients who were matched for post-mortem delay and also assessed neuropathologically prior to inclusion. The region of the brain used for these studies was the middle temporal cortex, area BA21 according to the Brodmann coordinates. We then used iTRAQ technology together with a multiplexing strategy which detected the fragmented ions of mass tags that generated a reporter ion in one of four different channels on the tandem mass spectra (Ross et al., 2004; Ong and Mann, 2005).

Figure 2 Work-flow of a typical neuroproteomics experiment.

Figure 2 Work-flow of a typical neuroproteomics experiment. The experimental design includes collection and selection of demographic matched case-control samples. The samples are first used for proteomic profiling in the discovery phase and then for biochemical study in the validation phase. The quantitative proteomic study can be performed using stable isotope labeling (as showed in the figure) or label-free methods. After data analysis and data mining, protein targets/candidates associated with disease are shortlisted for biochemical validation and generation of testable hypotheses for further functional studies. [Figure adapted from] *Proteomics*, 99, 54, 2014]

Use of quantitative proteomics to profile the human brain proteome presents clear advantages, since this discovery-driven approach can generate unforeseen insight into disease pathology which is likely to be overlooked by classical hypothesis-driven research (Lubec et al., 2003). The discovery-driven approach also has the advantage of being unbiased by prior assumptions, thus allowing the analysis and comparison between experimental conditions and data from thousands of individual peptides and proteins. The application of proteomics is usually divided into two primary fields, known as *profiling proteomics* and *functional proteomics* (Choudhary and Grant, 2004). Profiling proteomics consists of whole proteome analyses and determination of the changes that occur under specific experimental conditions, whereas functional proteomics characterizes the activity of the studied proteins, the interaction of these proteins with other molecules, and the PTMs that influence their properties. Application of iTRAQ quantitative proteomics to profiling of the brain proteome can provide data for both types of analysis, thereby maximizing potential to identify therapeutic targets among the underlying molecular mediators of neurodegenerative

diseases (Craft et al., 2013;Adav et al., 2014;Datta et al., 2014b;Gallart-Palau et al., 2015). Despite this ability to yield useful data on the molecular pathology of neurodegeneration, use of these techniques is complicated by the extreme complexity of CNS tissues, which restricts the characterization of low abundance proteins (Choudhary and Grant, 2004). Another drawback is the relative expense of the stable isotope labeling reagents and instrumentation required, which necessitates the pooling of samples from multiple individual subjects (Datta et al., 2010;Datta et al., 2011;Datta et al., 2014a;Datta et al., 2014b;Dutta et al., 2014). Yet another challenge associated with the use of quantitative proteomics to analyze brain tissues is the inability to assign the identified and characterized proteins to specific cell types in the affected tissues. Indeed, it is almost impossible to associate certain levels of an identified protein with a specific subpopulation of neurons (Craft et al., 2013). Finally, the implementation of robust statistics and the application of complementary validation methods are good strategies for maximizing the potential of proteomics to uncover pathological modifications of the brain proteome.

DPMs in neurodegenerative diseases

Oxidation and Nitration

Massive production of ROS in neurons and glial cells is a common hallmark of neurodegenerative diseases (Halliwell, 2006;Lim et al., 2014), and accordingly there are a vast number of brain tissue proteins that exhibit modulation by oxidative stress (Bayes and Grant, 2009). In the case of AD, the amyloid β -peptide 1-42 has been found to be highly oxidized in human brain, and can promote the oxidation of multiple other critical proteins both *in vitro* and *in vivo*, which may represent a key contributing factor to disease progression in AD (Butterfield et al., 2001;Selkoe, 2001;Butterfield, 2003) (Varadarajan et al., 2000;Butterfield and Lauderback, 2002). Proteins involved in neuronal excitotoxicity such as glutamine synthetase and glutamate transporter EAAT2 have also been found to exhibit significant oxidation in the human brain during AD, leading to significant loss of function in both proteins and the accumulation of glutamate in the extracellular domains (Masliah et al., 1996;Aksenov et al., 2001). The chaperone protein Hsp60 and the tubulin β -chain have similarly been revealed by proteomics to exhibit oxidation due to the action of the amyloid β -peptide 1-42 (Boyd-Kimball et al., 2005), and deterioration of these proteins has detrimental effects on cellular dynamics and proteostasis within the neurons of affected brain regions.

It is still unknown whether oxidative stress itself can trigger neurodegeneration or simply represents a byproduct of the complex neurodegenerative process. The pathology of PD begins at clusters of dopaminergic neurons in the midbrain nigrostriatal system, and it has previously been hypothesized that the dopamine molecule would have a predisposition to become oxidized. Indeed, normal enzymatic degradation of dopamine in dopaminergic neurons is known to generate hydrogen peroxide as a side-product (Adams et al., 1972;LaVoie et al., 2005). The protein α -Synuclein is highly oxidized and accumulates in oligomerized forms in PD brain tissues (Kruger et al., 1998), and the oxidized form has been detected in multiple different brain regions in patients with sporadic PD (Jenner and Olanow, 1998), suggesting that accumulation of this protein is a feature of vulnerable basal ganglia neurons.

The impact of oxidation on CNS protein function has been studied most completely for copper-zinc superoxide dismutase SOD1. This oxidized form of this mutant protein has been detected in the spinal cord of ALS patients, where SOD1 mutations account for around 10% of total diagnoses, and variable SOD1 oxidation has also been implicated involved in sporadic forms of ALS. Recent studies conducted in sporadic ALS patients using mass spectrometry observed that SOD1 exhibits oxidation of three crucial residues, cysteine 146, histidine 71 and histidine 120, which are essential for protein stability, structural folding, and enzymatic activity (Martins and English, 2014).

Protein tyrosine nitration (PTN) due to the effects of nitric oxide (NO) is a common event in CNS tissues (Schopfer et al., 2003; Lee et al., 2009). Accordingly, mass spectrometry has been used to identify an increased number of PTNs in the protein flotillin-1 α -tubulin in the brains of aging rodents (Dremina et al., 2005). The detrimental effects of protein nitration have been linked to diverse neurodegenerative diseases including AD, PD, HD and ALS (Beal, 2002; Schopfer et al., 2003; Sacksteder et al., 2006; Sawada et al., 2007). In addition, increases in protein PTNs are strongly associated with the presentation of oxidative stress in neurons and glial cells (Ischiropoulos and Beckman, 2003). Furthermore, the presence of protein PTNs also seems to be linked with neuroinflammation (Glass et al., 2010), and NO-induced PTNs in the amino terminal of the Tau protein have been reported to induce conformational changes and molecular modifications that promote tangle formation in the temporal regions of AD brain tissues (Reynolds et al., 2005).

In the case of PD, the protein α -Synuclein has been found to be nitrated at the tyrosine residues 39, 125, 133 and 136, leading to dramatically decreased solubility and protein aggregation (Takahashi et al., 2002). A SOD1 PTN induced by the enzymatic activity of the dismutase has also been reported in ALS, although nitration at tyrosine 108 does not appear to affect protein structure or function in this instance (Yeo et al., 2014).

Racemization and Isomerization

Racemization and isomerization are DPMs that occur at asparagine and glutamine residues and have been detected in isolated deposits in brain tissues from AD patients. Previous studies have shown that racemization of the aspartate residues in β -amyloid proteins can promote amyloidosis (Mori et al., 1994; Tomiyama et al., 1994). Several studies have now demonstrated that accumulation of isomerized residues in the amyloid protein of AD brains can promote fibrillation and aggregation, leading to increased protein toxicity (Roher et al., 1993a; Roher et al., 1993b). Some authors have since suggested that the study of amyloid DPMs should be expanded to encompass the analysis of succinimide, since this DPM is an intermediate product of aspartic acid dehydration and acts as precursor to isomerization and racemization (Orpiszewski and Benson, 1999; Orpiszewski et al., 2000).

Carbamylation

Protein carbamylation is not currently considered to be a major contributor to the neurodegenerative process, since this modification instead appears to exert neuroprotective effects in PD, AD and motor neuron diseases (MND) (Lapchak, 2008; Byts and Siren, 2009). In spite of these apparent beneficial effects, the molecular mechanism/s by which protein carbamylation protects against neural decline remains unknown and understudied. With

recent advances in mass spectrometry-based proteomic approaches, it should now be possible to uncover the function of carbamylation in neurodegenerative diseases, and to potentially harness these effects for the purposes of therapy. However, these promising beneficial effects of protein carbamylation in laboratory studies of neurodegeneration must be weighed against reports that the disease-promoting effects of harmful environmental factors such as smoking and uremia are also mediated by protein carbamylation (Wang et al., 2007).

Carbonylation

Previous studies have shown that induction of acute oxidative stress in the brain of *macaca fuscata* leads to up-regulation of carbonylated proteins, especially in the midbrain region (Oikawa et al., 2014). These findings are comparable to results obtained in PD brains in which an increase in protein carbonyls was detected in all brain regions (Alam et al., 1997). According to the conclusions drawn by Alam and colleagues (1997) in their study, the observed increase in carbonyl residues in the brain regions of PD patients cannot easily be attributed to the pathology of sporadic disease, since this could alternatively be explained by the pro-oxidant properties of the L-DOPA treatment that these patients receive. However, a subsequent proteomics study described the extent of protein carbonylation in AD brain tissues and reported that the proteins carbonic anhydrase II, syntaxin binding protein I, heat shock protein 70, and mitogen-activated protein kinase I were highly carbonylated in the brain regions first affected by AD (Sultana et al., 2010). In their report, the authors proposed that early carbonylation of these proteins could contribute to the early pathogenesis of AD. The importance of protein carbonylation in the pathology of neurodegenerative diseases is therefore clear, although it remains uncertain whether this modification is a consequence of the disease process or therapy given. Further studies will now be required to clarify this situation and to establish whether carbonylation is a feature of AD, the corresponding treatment, or even normal redox signaling processes in neurons (Suzuki et al., 2010).

Advanced glycation end products (AGEs)

AGEs has been traditionally been regarded as a hallmark of acute oxidative stress during neurodegeneration, but as yet there have been no functional consequences identified for this DPM. It was recently proposed that AGEs may represent a source of mitogenic factors for neurons and could promote reentry into the cell cycle (Schmidt et al. (2007);Kuhla et al., 2014). Neurodegeneration is associated with expansion of glial cells, which could potentially cause proliferation of AGEs in the affected brain regions as it has been shown by Sasaki and colleagues. These authors found that astrocytes shown positive signal for AGEs in brain regions devastated by the pathology of AD, furthermore, they and other authors suggest that a myriad of proteins may be modified by AGEs inside astrocytes during AD, and the details still remain unknown (Sasaki et al., 2001). Finally, we have to mention that the suppression of AGE formation by α -lipoic acid treatment has shown promising beneficial results *in vivo* during neurodegeneration (Kuhla et al., 2014).

Protein dysfunctions and cognitive impairment in neurodegenerative diseases

One of the most enigmatic phenomena in the pathology of neurodegenerative diseases is the appearance of clear and in some cases huge amounts of protein aggregates in neurons as

well as in the extracellular space. Whether these aggregates are linked to the cognitive deficits that characterize dementia and/or whether they are involved in the long-term degeneration of affected neurons is still a controversial matter. Protein misfolding is a natural process that is not normally pathological, since around 30% of the total proteins synthesized in healthy cells are misfolded and must be degraded via normal mechanisms (Schubert et al., 2000). In addition, there is accumulating evidence that almost all proteins in mammalian cells can form amyloid-like aggregates under the appropriate conditions (Mackay et al., 2001; Dobson, 2003; Fowler et al., 2006; Ionomidou et al., 2006; Fowler et al., 2007). Indeed, this hypothesis has been clearly established in yeast where inheritance of stable epigenetic traits is mediated by the transmission of cytoplasmic aggregated proteins (Serio and Lindquist, 2000). As we described above, the role of DPMs in the pathophysiological process of neurodegeneration remains elusive. Unbiased proteomic and systems biology studies of the neurodegenerative diseases are likely to generate valuable new data that can provide unforeseen insight into the molecular mechanism underlying these complex diseases. Recently, several studies have shown that sequestration of soluble toxic proteins in the cytoplasm to form insoluble aggregates can protect cells against cytotoxicity and reverses proteasome dysfunction (Bodner et al., 2006). Moreover it has been shown that in HD, cells that exhibit this type of inclusion die later than do cells that lack the aggregates (Arrasate et al., 2004; Schaffar et al., 2004). The harmful effects of protein aggregation may therefore depend on the extent of aggregation; when a cell is overloaded with oligomerized 'pathological' protein, it is possible that this initiates an abnormal autophagic flux state that allows small aggregates to be assembled into big aggresomes that significantly impair normal cell function (Chen et al., 2011; Swart et al., 2014). Current research on protein aggregation in neurodegenerative diseases now focuses more on the study of oligomeric intermediate proteins and their toxic effects (Conway et al., 2000; Glabe, 2006; Swart et al., 2014). The influence of spontaneous DPMs on oligomeric intermediate proteins has been scarcely studied at all, although some reports have implied that nitration and oxidation may increase the aggregation capacity of α -synuclein in PD (Xiang et al., 2013). There are currently no conclusive data on whether spontaneous non-enzymatic DPMs to oligomeric intermediates can modulate cellular toxicity in neurodegenerative diseases.

The term oligomeric intermediates essentially defines proteins with toxic properties and high probability to be sequestered or form aggregates in neurons. Generally, the proteins studied and defined as oligomeric intermediates in almost all studies are cytoplasmic proteins (α -synuclein and SOD1), and little is known about the dysfunctions that occur following oligomerization of other important synaptic proteins, ion channel proteins, and mitochondrial components etc.

Patients suffering from many of the neurodegenerative diseases including AD, PD, frontotemporal-dementia-ALS and HD manifest cognitive deficits that evolve into dementia. Cortical and subcortical dementias confer huge economic and social burdens on society, as well as severe consequences for the affected patients who may even lose their self-identity in the latter stages of disease (Hsiao et al., 2013). Degeneration of synapses is the first event in brains suffering from dementia, whereas the burden of senile plaques and amyloidosis in the brain does not correlate with dementia symptoms (Overk and Masliah, 2014). Accordingly, the extent of synapse loss correlates more strongly with the scores obtained in classical neuropsychological tests than do any other pathophysiological feature of disease

(DeKosky and Scheff, 1990;Sze et al., 1997;Sze et al., 2000;Serrano-Pozo et al., 2011). Given this apparent central role in neurodegenerative pathology, there is an urgent need to implement new studies based on unbiased approaches such as proteomics to discover the functional consequences of spontaneous DPMs on synaptic decay in dementia syndromes.

Deamidation of ion channel proteins Na(+)/K(+)-ATPase and dementia

The multiple functions of the ion channel protein Na⁺/K⁺-ATPase include the maintenance of the differential membrane potential in neurons, which is an essential feature of signal transduction process (Mobasheri et al., 2000). The pump-action mechanism of this protein also appears to mediate the transport of nutrients, glucose and other molecules along the axons and cytoplasmic membrane (Pavlov and Sokolov, 2000).

Figure 3 Schematic diagram of Na⁺ and K⁺ ion transport across Na⁺/K⁺ ATPase. [Adapted from J Proteome Research, 13(11), 4625, 2014]

Reduced expression levels of Na⁺/K⁺-ATPase have been reported in AD, PD and HD in both animal models and human brain tissues (Chauhan et al., 1997;Kumar and Kurup, 2002;Cannon, 2004). Other studies have also analyzed and associated the levels of Na⁺/K⁺-ATPase with severe neuronal loss in rat brains by using pharmacological inactivation of the protein (Bignami and Palladini, 1966;Lees and Leong, 1994). Since Na(+)/K(+)-ATPase is ubiquitously expressed in astrocytes and other brain cells, it was not possible until very recently to globally inhibit this protein and observe the effect on neuronal function (Harik,

1986;Renkawek et al., 1992). However, the use of the eye neuronal system of fruit flies (*drosophila melanogaster*) finally revealed that acute deficiency of this protein causes neuronal dysfunction, and that sustained-in-time deficit of Na(+)/K(+)-ATPase triggers progressive neurodegeneration in this model (Luan et al., 2014).

Although the link between dysregulation of Na(+)/K(+)-ATPase and progressive neurodegeneration appears well-established, dysfunctions in this protein had not been demonstrated in human dementia syndromes until 2014. Our recent iTRAQ quantitative proteomics study revealed deamidation in the asparagine and glutamine residues of Na(+)/K(+)-ATPase in the post-mortem brains tissues of VaD patients (Adav et al., 2014). Deamidation of proteins added a negative charge at the affected residue and could lead to beta-isomerization. The impact of protein deamidation has been extensively studied and reported by Noah E. Robinson (Robinson and Robinson, 2001a), who reported that deamidation of asparagine residues causes time-dependent changes in the charge profile and alters the three-dimensional conformation of several human proteins (Robinson and Robinson, 2001b;Robinson et al., 2001).

According to our analysis of VaD brain tissues, deamidation of Na(+)/K(+)-ATPase subunits in the evolutionarily conserved regions of the protein alters intracellular electrostatic interactions and leads to conformational changes at the Mg^{2+} , Cu^{2+} binding site. Three of the deamidated residues identified in our study were located close to critical components of the Na⁺/K⁺-ATPase structure that would be expected to compromise the ion transport activity of the protein (Adav et al., 2014). It has been previously been shown that mutation of Asn⁷¹⁵ to Ala can decrease the affinity of Na⁺/K⁺-ATPase for Mg^{2+} ions (Pedersen et al., 2000). Structural modelling experiments showed that the three deamidated residues present in VaD samples were located near to the catalytic site of this protein. In addition, modification of residues 210 and 220 has been proposed to cause defects in normal protein phosphorylation and dephosphorylation mechanisms, leading to altered ATP hydrolysis. The identification of these DPMs in the Na⁺/K⁺-ATPase ion channel were successfully uncovered by proteomic profiling, but since they have yet to be evaluated using alternative approaches, the roles played by these DPMs in VaD pathogenesis are currently unknown. Further application of unbiased proteomics approaches to the study of dementia syndromes is likely to provide unprecedented detail about the molecular pathology of neurodegenerative diseases.

Figure 4 Structural modeling of the potential impact of deamidation sites on the biological function of the Na⁺/K⁺ ATPase ion channel protein [Adapted from J Proteome Research, 13(11), 4625, 2014].

Deamidation of synaptic proteins and dementia

Synaptic failure is the most common pathophysiological characteristic shared by both VaD and AD (Kalaria, 2000;Kalaria, 2002;Iadecola, 2013). Accordingly, loss of synapses has been reported as one of the most significant contributors to the cognitive impairments manifest in VaD and other neurodegenerative diseases. It has also been shown that following synaptic loss, the remaining synapses alter their shape as a potential compensatory mechanism (Scheff et al., 1990;Scheff, 2001;Scheff et al., 2007;Sisková et al., 2010). However, the vast majority of studies performed in this field to date have been focused on the structural analysis of degenerating neuronal synapses using only imaging techniques. Consequently, the molecular profile associated with synaptic failure in VaD remains largely unknown. Our group pioneered the study of DPMs, dysfunctions and deregulations in synaptic proteins in

human VaD (Gallart-Palau et al., 2015). According to our quantitative findings using iTRAQ proteomics to profile human VaD post-mortem brain tissues, synaptic immunoglobulins were among the most perturbed proteins in VaD temporal cortices, whereas synaptic proteins such as Bassoon and SNAP25 were substantially up-regulated. In contrast, VaD brain tissues also displayed marked down-regulation of the synaptic proteins AP2 and clathrin heavy chain 1, which are required for clathrin-mediated endocytosis.

When we extended our study to the analysis of synaptic protein deamidation in VaD temporal cortices, we observed that the protein synapsin1 displayed significant accumulation of deamidated asparagine and glutamine residues when compared with age-matched controls (Gallart-Palau et al., 2015). We next used the I-Tasser server to perform *ab initio* protein modelling (Zhang, 2008; Roy et al., 2010; de Carvalho and De Mesquita, 2013) which enabled us to construct models of both wild-type synapsin1 and the deamidated form which was detected in VaD tissues. Next we aligned the structure of the deamidated synapsin1 to the physical templates of wild-type synapsin1 (obtained by nuclear magnetic resonance using the software TM-align (Zhang and Skolnick, 2005)). This then allowed to assess the likely impact of VaD-associated deamidations on the structure and function of the protein, and confirmed that the deamidation sites in synapsin1 were likely to induce pathological changes in protein conformation.

Figure 5 Structural modeling of the potential impact of deamidation sites on the conformation of synapsin I protein [Adapted from Neurochemistry International, 80, 87, 2014].

The spontaneous protein deamidation events and other DPMs identified in dementia and neurodegeneration so far represent only the tip of the iceberg. Further studies using unbiased proteomics and systems biology approaches will now be needed to provide novel insight into the role of protein dysfunction in neurodegeneration, and should finally

establish whether DPMs are merely the consequence of the ongoing neurodegenerative processes or are in fact essential mediators of these pathologies.

Concluding Remarks

The study of protein dysfunction in CNS tissues dates back more than 150 years, but progress in understanding the role of protein dysfunction in the pathology of neurodegenerative diseases has required substantial advancement in biochemical analytical methods and techniques. Mass spectrometry-based proteomics is an unbiased systems biological technique that enables the study of protein composition, characteristics and interactions in both healthy tissues and pathological conditions. Application of mass spectrometry-based proteomics to the study of neurobiology has already facilitated numerous significant discoveries with the potential for therapeutic applications, as we have detailed in this chapter. However, it is the unforeseen insight that proteomics may provide in future years into the molecular basis of neurodegenerative diseases that could deliver radically improved patient outcomes in the clinic.

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