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REVIEW ARTICLE

Advances in human brain proteomics analysis of neurodegenerative diseases

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ABSTRACT

Neurodegenerative diseases are characterized by progressive loss of neurons manifested as motor dysfunction and/or cognitive decline. Aberrant protein aggregation with altered physicochemical properties occurs in most neurodegenerative diseases. The pathophysiological mechanisms leading to the onset and progress of neurodegenerative diseases are still not fully understood. On the one hand, limited studies investigate neurodegenerative disease from human brain tissues. On the other, a comprehensive and efficient analysis method is needed to detect the signaling pathways evolved in neurodegenerative disease. Proteomics on human brains identifies key diagnostic biomarkers and treatment/therapeutic targets of neurodegenerative disorders. In recent years, several proteomics studies conducted on brain tissues from patients with neurodegenerative diseases have shown that changes in protein abundance or post-translational modification underly the disease pathogenesis. In this review, we summarize the major advances of human brain proteomics in the research on Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis and Huntington's disease as the most common neurodegenerative diseases. Finally, we proposed some perspective clues for future work.

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Introduction

The brain is one of the most complex organs in mammals. As the central control organ, brain homeostasis and signal transduction are maintained by the combined action of various cells. Revealing the secrets of the human brain is perhaps one of the most significant scientific challenges of the 21st century. Since the Human Brain Project (HBP) initiation in 1997, massive interdisciplinary scientific collaborations have been undertaken to develop in-depth insights into the human brain and related diseases (Amunts et al., 2016; Amunts et al., 2019). In recent years, the emergence of formal 'brain banks' (e.g., the UK Brain Archive Information Network (Nicoll, Bloom, Clarke, Boche, & Hilton, 2022) and the Chinese Human Brain Bank (B. Xu et al., 2016)) have also provided shared resources and reliable platforms for extensive research in neuroscience and associated disorders.

Neurodegenerative diseases are a subset of fatal diseases that have increased in prevalence with the expansion of the aging population in recent years (Heemels, 2016). Thus, neurodegenerative diseases represent a significant threat to human health. While animal models of human neurological diseases help us to understand the dynamics of biological processes and cause-and-effect relationships, a disease model does not replicate the disease process in humans and the limitations of animal models should be taken into consideration when interpreting results (Burrows et al., 2019; Chia, Tan, & Chao, 2020; Dawson, Golde, & Lagier-Tourenne, 2018; Franco Bocanegra, Nicoll, & Boche, 2018; Nicoll et al., 2022; Swarup & Julien, 2011). Direct studies on human brain tissue could greatly improve our understanding of neurological diseases' complex mechanisms. Proteomic analysis of brain tissue is a powerful and promising technique that has been used successfully to identify novel biomarkers and candidate therapeutic targets. Innovations have accelerated discoveries in the field of proteomics have accelerated discoveries in the field of proteomics in sequencing technologies based on mass spectrometry (MS). Furthermore, evolving multi-omics approaches offer indispensable, systematic tools for elucidating the biomolecular mechanisms of diseases. In the last few decades, several proteomics studies on brain tissues from patients with the neurodegenerative disease have indicated that a broad spectrum of biochemical pathways underlies the disease progression.

In this review, we provide a brief overview of the recent human brain proteomics studies in Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis, amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD) as the most commonly encountered neurodegenerative diseases.

An overview of proteomics technology

Proteomics is the large-scale study of proteins in organisms encoded by their genomes and can be performed to identify potential biomarkers and elucidate mechanisms of pathophysiology (McArdle & Menikou, 2021). As many neurodegenerative diseases are described as proteinopathies, proteomics is thought to be an ideal technology to elucidate the aberrant protein expression in disease-affected human brain tissues. Proteomics analyses are usually performed using various technologies, such as chromatography-based techniques, enzyme-linked immunosorbent assay (ELISA), western blotting, and gel electrophoresis techniques. In addition, MS, which is used to identify specific chemicals and molecules, is commonly applied in proteomics studies since this technique can be used to assess protein abundance, post-translational modifications (PTMs) and protein interactions. However, proteins were not commonly analyzed by MS until before the development of two ionization methods in the late 1980s (Griffiths, 2008): electrospray ionization (ESI) (Fenn, Mann, Meng, Wong, & Whitehouse, 1989) and matrix-assisted laser desorption ionization (MALDI) (Tanaka et al., 1988). In general, three significant steps are included in a proteomics study (**Fig. 1A-C**): (i) sample processing, (ii) MS processing, and (iii) bioinformatic analysis, with each step providing a diverse range of strategies to achieve the analytical goals of protein identification and quantification.

Almost any biological samples can be used for proteomics research so long as they can be ionized for MS. Human brain tissues for proteomics studies are commonly derived from postmortem examinations (autopsies) and minor specimens taken from the living during surgical procedures (i.e., biopsies and resections) (Nicoll et al., 2022). It is noteworthy that sample collection, storage and treatment should be handled using uniform standards, which helps to obtain repeatable results and avoid analytical biases (Comes et al., 2018; Virginie Licker & Burkhard, 2014). Postmortem interval (PMI) should also be considered when performing postmortem brain proteomics since many proteins will degrade during a long delay (Virginie Licker & Burkhard, 2014; V. Licker, Kövari, Hochstrasser, & Burkhard, 2009). Thus, a short PMI is thought to be of vital importance and should generally improve confidence in brain proteomics results.

Currently, two general approaches, known as top-down and bottom-up, are used for quantitative proteomic analysis. The top-down approach is used to analyze full-length proteins. One of the advantages of this strategy is that it can recognize various proteoforms, including proteolytic fragments, phosphorylated peptides and post-translationally modified proteoforms (Catherman, Skinner, & Kelleher, 2014; Donnelly et al.,

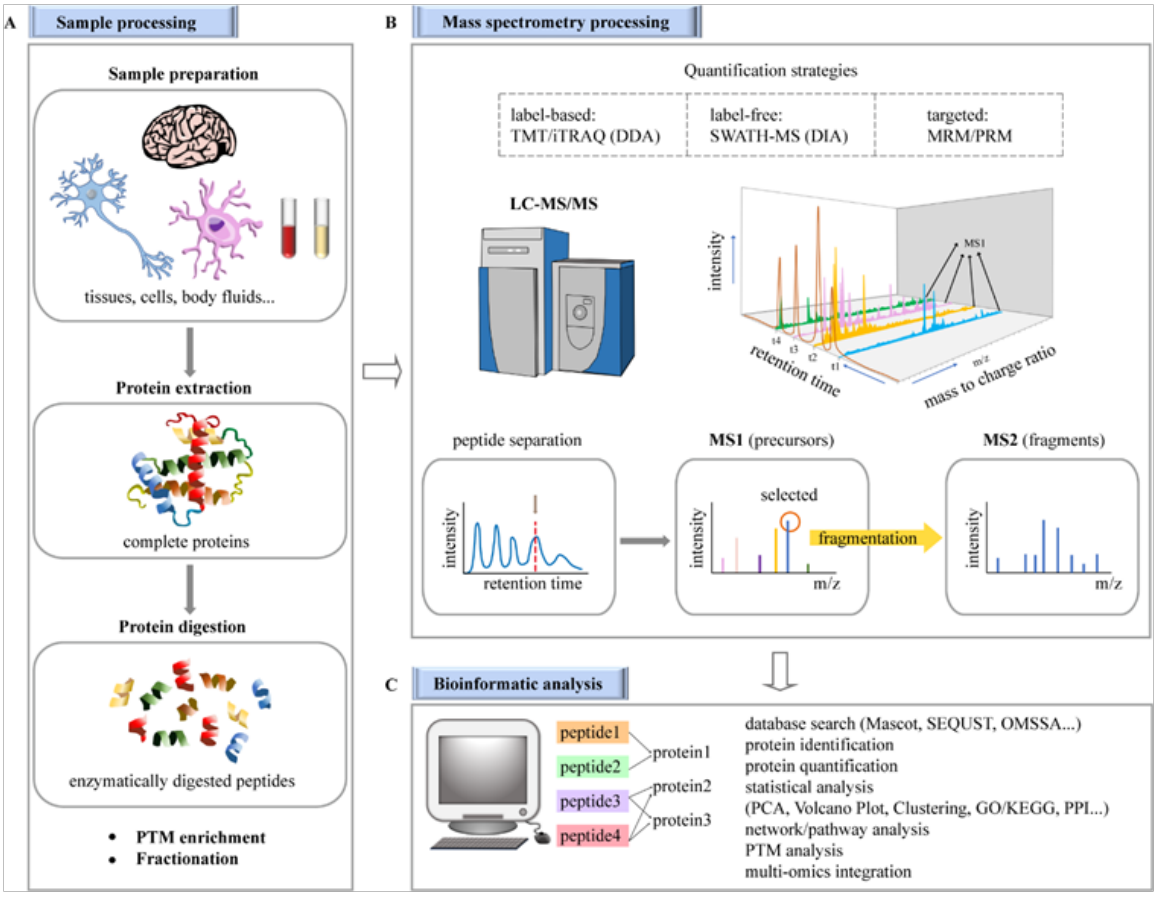

Figure 1. MS-based quantitative proteomics workflow. **(A)** Sample processing. Proteins used for proteomics can be acquired from various biological samples, such as tissues, cells or body fluids. The total proteins obtained can be used directly for downstream detection (the 'top-down' approach) or digested before analysis (the 'bottom-up' strategy). The enrichment step is important for PTM analysis, as the abundance of PTM proteins is relatively low. As an optional step, sample fractionation can be achieved by high-performance liquid chromatography (HPLC), which increases the detection of low abundance peptides. **(B)** Mass spectrometry processing. Protein quantification can be achieved by several methods, such as label-based methods (e.g., TMT/iTRAQ, which usually involves DDA analyses), label-free quantification (LFQ) approaches (e.g., SWATH-MS, which relies on DIA analyses), and targeted methods (e.g., MRM/PRM). By performing LC-MS/MS, proteins/peptides are first separated by liquid chromatography, and the eluted peptide species are analyzed by sequentially mass spectrometry (MS1) before several precursors are selected, fragmented and sequenced for subsequent mass spectrometry analysis (MS2). **(C)** Bioinformatic analysis. Protein databases are searched to identify proteins from sequenced peptides. After protein identification and quantification, further analyses, such as PCA, volcano plot analysis, clustering analysis, GO/KEGG, and protein interaction (PPI), can be conducted in silico. Abbreviations: PTM, post-translational modification; TMT, tandem mass tag; iTRAQ, isobaric tags for relative and absolute quantification; DDA, data-dependent acquisition; DIA, data-independent acquisition; SWATH-MS, sequential windowed acquisition of all theoretical mass spectra; MRM, multiple reaction monitoring; PRM, parallel reaction monitoring; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PCA, principal components analysis; GO/KEGG, Gene Ontology/Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction.

2019). It is also an excellent choice to identify modifications of a single protein, but requires some starting samples. However, the top-down strategy is challenging due to the lack of proteome-wide coverage, sensitivity and high throughput capacity (Murtaza, Uy, & Singh, 2020).

In contrast, the bottom-up approach (called shotgun proteomics) relies on analyzing peptides digested from full-length proteins. Smaller peptides are biochemically homogenous and easier to analyze, allowing more accurate identification of sequences (Chait, 2011). This proteomics approach has the advantages of high proteome coverage and rapid scanning rates, while its disadvantages include incomplete protein sequence coverage and loss of labile PTMs (Yates, Ruse, & Nakorchevsky, 2009). The bottom-up strategy is commonly applied in MS-based proteomics because of its high sensitivity, throughput, robustness and proteome coverage.

The significant streams for quantitative proteomics can also be divided into two categories: label-free quantification (LFQ) and label-based methods. Due to the ability to run multiple samples without limitation, the LFQ method is excellent for identifying changes in various cell and tissue types by either data-dependent acquisition (DDA) or data-independent acquisition (DIA) analyses. Notably, a recent LFQ approach known as the sequential windowed acquisition of all theoretical mass spectra (SWATH-MS) has been widely utilized for analyzing complex mixtures by DIA (Ludwig et al., 2018). On the other hand, chemical or metabolic tags, such as isobaric tags for relative and absolute quantification (iTRAQ) and tandem mass tag (TMT), are widely used in label-based proteomics. This approach can reduce noise and run times and is generally operated in the DDA mode. Targeted techniques, like multiple reaction monitoring (MRM) and parallel reaction monitoring (PRM), are also used for protein quantification. Liquid chromatography (LC) is commonly paired with MS methods to analyze more complex protein/peptide mixtures with higher specificity. Thus, even small biological samples can be analyzed to screen large numbers of proteins. Moreover, tandem-MS (MS/MS) is typically used in bottom-up proteomics, with LC-MS/MS as the most common method used to quantify proteins on a global scale. Peptide species eluted from LC columns are directly ionized into the mass spectrometer for analysis, where precursors can be selected and fragmented for further peptide identification.

Using various bioinformatics tools, accurate information can be extracted from raw MS data to allow protein identification and quantification. Today, many large-scale protein databases and MS spectral libraries are available for academics with commercial or free access. Furthermore, numerous specialized software packages and algorithms are available for post-MS bioinformatical analysis. After protein identification and quantification, several methods can be used to test further hypotheses (Bai et al., 2021), including statistical classification, differential expression, network/pathway analysis, multi-omics integration, etc.

Overall, proteomics not only allows the identification and quantification of proteins but also provides information on protein localization, post-translational modifications, functions and interactions. Several excellent proteomics investigations of neurodegenerative diseases have been reported based on human fluid samples, animal models or cellular models, while similar studies on human brain samples are relatively rare. In the following review, we focus on proteomics studies of brain tissues from patients with the most common neurodegenerative diseases.

Alzheimer's disease

Alzheimer's disease (AD) is the most common neurodegenerative disease, with its prevalence increasing with age (Hou et al., 2019). AD is thought to be a progressive disease, and noticeable symptoms such as cognitive decline, memory impairments and language problems arise only after 20 years or more of brain changes ("2021 Alzheimer's disease facts and figures," 2021; Bai et al., 2021). Currently, more than 55 million people worldwide have been diagnosed with AD and other dementias, and the number is projected to reach 152 million by the year 2050, with increased psychosomatic and financial burden on individuals, families and the wider public (Gauthier, Rosa-Neto, Morais, & Webster, 2021; International, 2019). Typical pathological manifestations of AD comprise the extracellular deposition of amyloid β protein (Aβ) derived from β-amyloid precursor protein (APP) cleavage and hyperphosphorylated microtubule-associated

protein Tau (MAPT) in intracellular neurofibrillary tangles (NFTs). These signs are considered essential for the neuropathologic diagnosis of AD and the categorization of its stage (DeTure & Dickson, 2019; Hyman et al., 2012; Tiwari, Atluri, Kaushik, Yndart, & Nair, 2019). Although the amyloid and tau hypotheses are widely accepted (Ballatore, Lee, & Trojanowski, 2007; Hardy & Higgins, 1992; Selkoe, 1989; Selkoe & Hardy, 2016), a myriad of alternative hypotheses are increasingly gaining traction, like the mitochondrial hypothesis (Kerr et al., 2017; W. Wang, Zhao, Ma, Perry, & Zhu, 2020), inflammatory hypothesis (Kinney et al., 2018; Wyss-Coray & Rogers, 2012) and cholinergic hypothesis (Hampel et al., 2018).

The application of laser capture microdissection (LCM), a technique for dissecting and isolating a tissue region of interest, provides essential insights into AD plaque pathology. In 2004, Liao and colleagues examined proteome changes in the amyloid plaques (APs) isolated from postmortem AD brain tissues by LCM (Liao et al., 2004). 488 proteins were identified, including 26 differentially expressed (DE) proteins enriched in the APs compared with the levels detected in the surrounding non-plaque tissues. The findings of the pilot proteomic analysis confirmed the presence of most proteins detected previously in APs by immunohistochemistry (Atwood, Martins, Smith, & Perry, 2002). In 2017, Drummond and coworkers analyzed the protein differences in APs between rapidly progressive AD (rpAD) and typical sporadic AD (sAD) patients by combining LCM with label-free quantitative LC-MS/MS (Drummond et al., 2017). Compared to sAD plaques, the rpAD plaques contained significantly lower levels of astrocytic proteins and higher levels of neuronal proteins, particularly key presynaptic proteins (such as SNAP25, syntaxin-binding protein 1, syntaxin 1A and piccolo). Furthermore, altered proteins in rpAD plaques were significantly enriched in several pathways, including actin cytoskeleton signaling, Rho family GTPase signaling, and virus entry via the endocytic and phagosome maturation pathways. Furthermore, LCM coupled with TMT high-throughput MS was utilized to characterize the proteomes of APs in human AD and age-matched non-AD brains (Xiong, Ge, & Ma, 2019). Approximately 40 proteins were found to be enriched in both AD and non-AD APs (e.g., apoE, midkine, TMEFF2, netrin-1, VGFR1, C1q and C4). Some proteins were upregulated only in AD APs (including synaptic structural proteins, C1r, C1s, and C5-C9), indicating that the major components of AD and non-AD APs are similar. At the same time, there are differences in the activation of the complement system in these two APs.

Current MS-based strategies enable either focused or proteome-wide analysis of PTMs in AD specimens. Notably, tau aggregation is associated with extensive PTMs, including phosphorylation, ubiquitination, acetylation, methylation, glycosylation, sumoylation, oxidation and cleavage (Bai et al., 2020; Dujardin et al., 2020; L. Martin, Latypova, & Terro, 2011; Y. Wang & Mandelkow, 2016; Wesseling et al., 2020). Using an immunoaffinity approach coupled with label-free MS-based proteomics, Abreha et al. mapped the ubiquitin-modified proteome profile in postmortem AD brains, identifying 4,291 unique ubiquitylation sites in 1,682 proteins, among which more than 800 ubiquitylation sites were altered in AD (Abreha et al., 2018). Furthermore, changes in ubiquitylation patterns in AD brain implied that proteostasis mechanisms are at play in the pathogenesis of AD. In addition, Arakhamia and coworkers utilized MS approaches and cryo-electron microscopy (cryo-EM) to compare the structures and PTMs of tau filaments from human brain tissue with cortico-basal degeneration (CBD) and AD (Arakhamia et al., 2020). This research indicated that ubiquitination of tau could influence filament structure and mediate fibril diversity. By combining the multiplexed TMT strategy with two-dimensional LC/LC-MS/MS, Bai and colleagues determined the profiles of the whole proteome and phosphoproteome in AD progression (Bai et al., 2020). In total, 34,173 phosphosites on 7,083 phosphoproteins were identified, with microtubule-associated tau as the most elevated phosphoprotein in AD. Further integrated multi-omics revealed pathways associated with AD, including the amyloid cascade, inflammation, complement activation, WNT signaling, TGFβ/MBP signaling, lipid metabolism, iron homeostasis, and membrane transport.

Different brain regions show distinct pathologies during the progression of AD. Overall, the hippocampus (HP), entorhinal cortex (ENT)

and cingulate gyrus (CG) are the parts of the brain known to be heavily affected by AD. In contrast, the sensory cortex (SCx) and motor cortex (MCx) are less seriously affected regions, and the cerebellum (CB) is relatively 'spared'. Xu and colleagues used MS-based approaches to analyze postmortem tissue samples from each of these six regions of the brain of patients with AD and matched controls and revealed the changes in protein expression in the specific area of brain regions during AD progression (J. Xu et al., 2019). A total of 4,835 distinct proteins were quantified in at least one brain region, among which 1,899 proteins were common to all six regions. Unsurprisingly, the severely affected regions (HP, ENT, and CG) showed the most significant number of changes in protein expression (around 30% of the quantified proteins). In contrast, the less affected areas (MCx, SCx) showed changes in only 11%–13%. Strikingly, the supposedly 'unaffected' CB displayed an unexpected pattern of protein expression changes distinct from other brain regions, indicating that these proteins may be protective. Signaling pathways involved in apoptosis and cell cycle regulation, including the HIPPO, ERK/MAPK, PI3K/AKT and Wnt/β-catenin pathways, were widely dysregulated in severely affected brain regions. In addition, four candidate genes (*STXBP1*, *CRMP1*, *ACTR10* and *AMPH*), which may be critical regulators of protein expression in AD pathogenesis, were identified by correlation network analysis. Furthermore, astrocyte-associated region-consistent DE proteins and oligodendrocyte-associated region-specific DE proteins were identified by proteomics analysis of hippocampal subfields and the entorhinal cortex region obtained from postmortem AD brain specimens, indicating the critical role of glial cells in AD pathogenesis (Gao et al., 2022). In a recent study, Johnson and coworkers analyzed 516 dorsolateral prefrontal cortex tissues from asymptomatic and symptomatic AD cases and controls by TMT-MS-based quantitative proteomics (E. C. B. Johnson et al., 2022). The weighted gene co-expression network analysis (WGCNA) algorithm (Langfelder & Horvath, 2008) was used to construct the AD-related protein co-expression network consisting of 44 protein co-expression modules. Notably, twelve modules or module families, including post-synaptic density, matrisome, cell-extracellular matrix (ECM) interaction, mitogen-activated protein kinase (MAPK) signaling and metabolism, glycosylation/endoplasmic reticulum (ER), oligodendrocyte/myelination, RNA splicing, synapse/neuron, ubiquitination, mitochondria, sugar metabolism and protein transport, were identified as the most strongly correlated to AD traits. In addition, evaluation of the cell type nature of each module based on cell-type-specific protein markers supported the contribution of diverse cell types (neuron, astrocyte, microglia, oligodendrocyte and endothelia) to AD pathogenesis (E. C. B. Johnson et al., 2020).

Overall, human brain proteomics analyses allow for an unbiased exploration of potential biomarkers and pathways that underlie the pathogenesis of AD (**Fig. 2**). Accumulating evidence supports the notion that amyloid β (Aβ) plaques and NFTs are pathological hallmarks of AD. Aβ plaques develop with altered cleavage of APP, resulting in mitochondrial dysfunction and oxidative damage eventually leading to neuronal damage and death. Moreover, excessive Aβ accumulation leads to the activation of several kinases (primarily GSK3β and CDK5), causing tau hyperphosphorylation and the formation of NFTs (Tiwari et al., 2019). Consequently, fibrillary and insoluble NFTs disturb the communication between neurons and contribute to neurotoxicity. Several AD-related pathways have also been reported, including neuroinflammation, complement, astrocyte/microglial metabolism, actin cytoskeleton signaling, WNT signaling, MAPK signaling and metabolism, RNA splicing, apoptosis and membrane transport. Future research targeting these pathways may advance therapeutic progress in AD.

Extracellular Aβ plaques and intracellular NFTs are typical pathological manifestations of AD that can lead to neuronal damage and death. Several pathways have been implicated in the pathogenesis of AD, including mitochondrial function, neuroinflammation, the complement system, astrocyte/microglial metabolism, MAPK signaling and metabolism, actin cytoskeleton signaling, apoptosis, RNA splicing, WNT signaling and membrane transport. The methods used for analysis are shown in blue.

Parkinson's disease

Parkinson's disease (PD), the second most common neurodegenerative disease, is associated

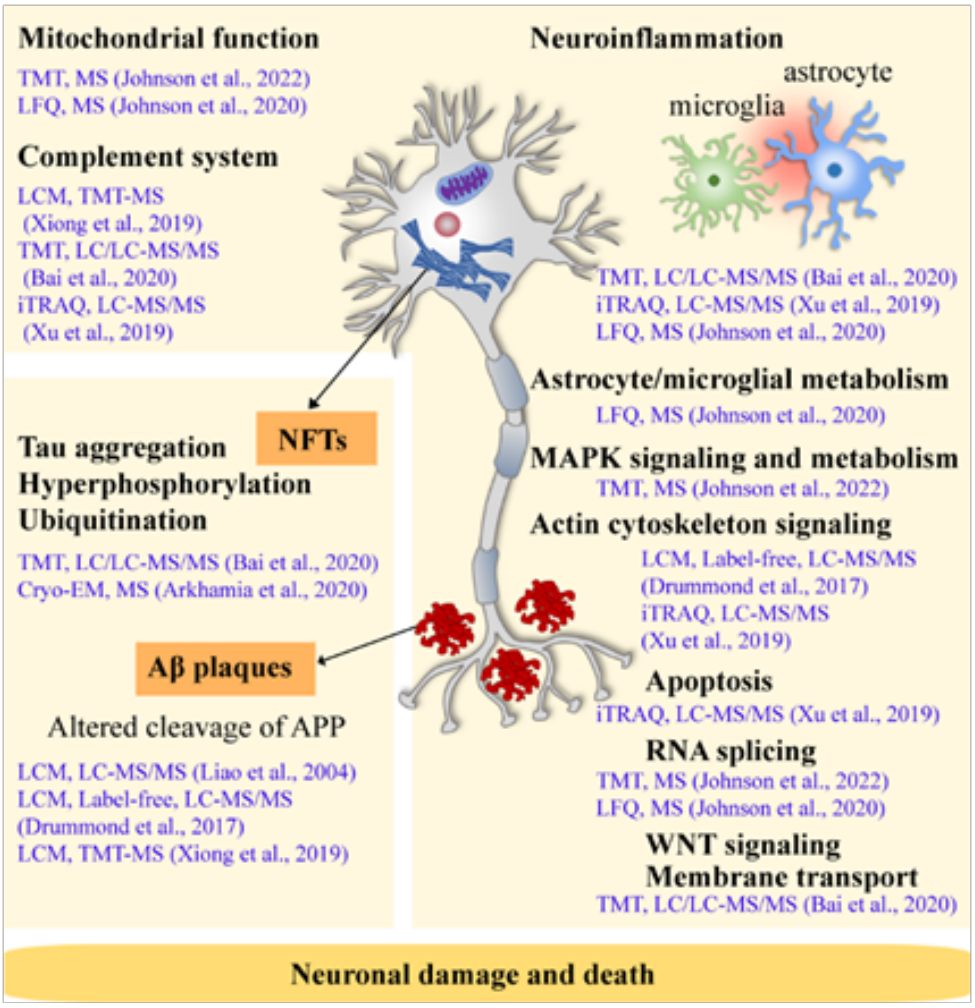

Figure 2. Pathways implicated in the pathogenesis of AD according to human brain proteomics studies.

with a variety of motor symptoms, such as bradykinesia, resting tremors, rigidity and changes in posture and gait (Dixit, Mehta, & Singh, 2019; Erkkinen, Kim, & Geschwind, 2018; Tolosa, Garrido, Scholz, & Poewe, 2021). PD is reported to affect more than 6 million individuals worldwide ("Global, regional, and national burden of neurological disorders, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016," 2019), and the number is projected to exceed 12 million by 2040 (Dorsey & Bloem, 2018). The incidence of PD increases with age and rises sharply at around age 65. Other risk factors have also been shown to play a role in the pathogenesis of PD (e.g., increasing longevity, declining smoking rates, and the by-products of industrialization) (Dorsey, Sherer, Okun, & Bloem, 2018). As the pathological hallmark of PD, Lewy bodies (LBs) consist of aggregates of misfolded or abnormal α-synuclein (SNCA) and other proteins, with dopaminergic neuronal loss in the substantia nigra (SN) and other brain regions (Dixit et al., 2019; Erkkinen et al., 2018; Tolosa et al., 2021).

Detailed proteomics analyses of brain regions involved in PD pathology are crucially important for elucidating the underlying mechanisms , and identifying potential biomarkers. Proteomics studies of the SN of postmortem PD brain revealed the involvement of several pathogenic processes in PD pathogenesis, including mitochondrial dysfunction, oxidative stress, cytoskeleton impairment, intracellular transport processes, synaptic activities, energy metabolism, protein aggregation, and inflammation (V. Licker

et al., 2012; V. Licker et al., 2014). A subsequent proteomics study in which 2D gel electrophoresis and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF-TOF) analyses were employed revealed alteration in the protein expression profiles of the SN and ventral tegmental area (VTA) from postmortem PD brains (Dutta et al., 2018). This study indicated that prohibitin, an essential protein involved in the maintenance of mitochondrial integrity, may regulate dopaminergic cell death in SN and protect neuronal cells in the VTA during the progression of PD. Although dopaminergic neurons in the SN are highly susceptible to PD, olfactory dysfunction is present in up to 95% of PD patients and is considered one of the earliest symptoms (Attems, Walker, & Jellinger, 2014; Doty, 2012). In 2019, Lachén–Montes and colleagues combined MALDI imaging mass spectrometry (MALDI-IMS) and label-free quantitative proteomics to explore the olfactory bulbs (OB) proteome in postmortem brain from clinically confirmed PD cases, and identified more than 250 DE proteins between PD specimens and controls (Lachén-Montes et al., 2019). Furthermore, protein interaction networks revealed modulation of the ERK1/2, MKK3/6 and PDK1/PKC signaling pathways, indicating the critical role of the OB in PD pathogenesis and highlighting their importance as potential novel therapeutic targets. In another study, Dumitriu et al. combined proteomics with RNA-sequencing transcriptomics to investigate loci implicated in genome-wide association studies, offering novel insights into the alteration of the prefrontal cortex in PD (Dumitriu et al., 2016). In total, 283 DE proteins were identified in PD cases compared to controls, involving confirmed proteins (e.g., SNCA, GAD1 and NPTX2) and other novel candidates. Subsequent functional enrichment analyses based on proteomics data supported the involvement of mitochondrial dysfunction in PD pathology, which was consistent with previous studies (V. Licker et al., 2012; Mullin & Schapira, 2013; Riley et al., 2014). The study did not show any marked correspondence between proteins and mRNA levels identified by proteomics and RNA-sequencing, respectively. In a recent study, Villar–Conde et al. reported the hippocampal proteome in patients with PD and revealed 83 DE proteins (Villar-Conde et al., 2021). Among them, several proteins were associated with synaptic structures, including downregulated (caskin-1, TMEM163 and REEP2) and upregulated (δ2-catenin, AHA-1, PHYHIPL and α-1-syntrophin) proteins, suggesting that hippocampal synaptic dysfunction plays a role in PD. However, minimally invasive samples (i.e., serum and plasma samples) are predominantly used to evaluate proteome profiles in PD, with the results highlighting the importance of coagulation, inflammation, and oxidative stress pathways (Mayo, Benito-León, Peña-Bautista, Baquero, & Cháfer-Pericás, 2021). In addition, in comparison with healthy participants, PD patients showed differences in the levels of some proteins, such as CRP, interleukins, necrosis factors, transferrin, glia fibrillary acidic protein (GFAP), neurofilament proteins, Rab35, ApoA1, and DJ-1. Although these differences require further clinical verification, these proteins may serve as diagnostic biomarkers of PD (Akıl et al., 2015; An, Pu, Xiao, & Zhang, 2018; Chiu et al., 2016; García-Moreno et al., 2013; R. Kim et al., 2018; Kitamura et al., 2018; Si et al., 2018; Song, Chung, Kim, & Lee, 2011; Su, Chen, Li, & Wu, 2012; Umemura et al., 2015; Yamagishi et al., 2018).

Although existing research has yielded some information, the pathophysiological mechanisms underlying PD are still not fully understood. Current human brain proteomics studies have confirmed the involvement of well-known pathways such as mitochondrial dysfunction and oxidative stress in PD pathogenesis but also point toward other mechanisms, such as inflammation, protein aggregation, cytoskeleton impairment, intracellular transport processes, synaptic activities and energy metabolism. These disease mechanisms eventually lead to dopaminergic cell dysfunction and death (**Fig. 3**). Notably, SNCA plays a crucial role in these processes and has been shown to cause direct mitochondrial toxicity (Mullin & Schapira, 2013). Thus, complete elucidation of the mechanisms underlying its aggregation and degradation may provide therapeutic targets for PD.

Intracellular Lewy bodies (LBs) are the pathological hallmark of PD and have a causal role in neuronal loss. Several studies have confirmed the importance of mitochondrial dysfunction and oxidative stress in PD pathogenesis. Other pathways implicated in PD include inflammation, protein aggregation, cytoskeleton impairment, intracellular transport processes, synaptic dysfunction and energy metabolism. The methods used for analysis are shown in blue.

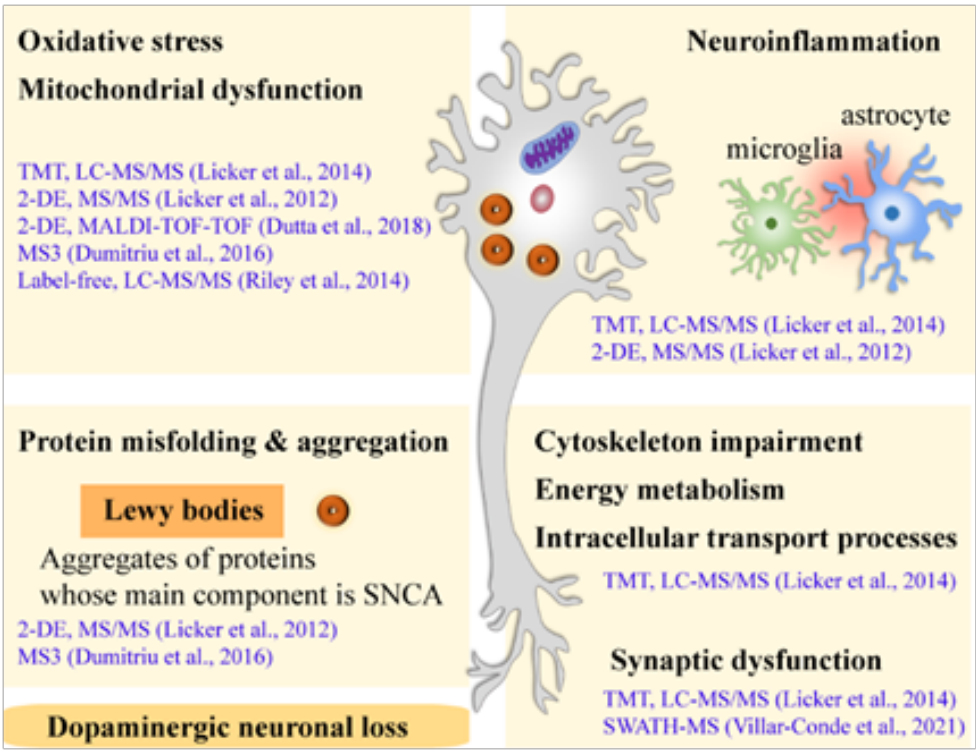

Figure 3. Proposed pathogenic pathways involved in PD according to human brain proteomics studies.

Multiple sclerosis

Multiple sclerosis is a chronic autoimmune demyelinating and neurodegenerative disease of the central nervous system (CNS) that mainly affects young adults (20–45 years) (Dobson & Giovannoni, 2019). It is estimated that approximately 2.8 million people worldwide have multiple sclerosis, and females are more susceptible (Walton et al., 2020). Genetic (e.g., HLA DRB1*15:01), environmental (e.g., vitamin D), and lifestyle (e.g., smoking) risk factors that contribute to the development of multiple sclerosis have been identified (Thompson, Baranzini, Geurts, Hemmer, & Ciccarelli, 2018). The pathological hallmarks of multiple sclerosis are inflammatory infiltration, demyelination, and neurodegeneration in the brain, spinal cord, and optic nerves (Olek, 2021; Thompson et al., 2018). Peripheral immune cells (e.g., T and B cells) are thought to enter the CNS through the damaged blood-brain barrier (BBB) (Ortiz et al., 2014), where they recognize antigens (probably autoantigens) and initiate inflammatory responses by producing pro-inflammatory cytokines and recruiting more immune cells to the lesion (Lassmann & Bradl, 2017; Sandi et al., 2022). Demyelination then occurs as a result of inflammation. Axons are relatively preserved in the early phases; however, once the remyelinating capacity is exhausted, irreversible axonal injury develops, eventually leading to neurodegeneration (Sandi et al., 2022) (**Fig. 4**).

In 2021, Sen et al. reviewed 29 studies published between 2004 and 2019 that used proteomic approaches to assess multiple sclerosis patient samples to identify critical molecular pathways related to the identified canonical proteins (Sen, Almuslehi, Shortland, Mahns, & Coorssen, 2021). Of the 29 studies, only two (Broadwater et al., 2011; Ly et al., 2011) analyzed postmortem brain tissue samples, whereas the rest of the studies predominantly used either CSF or blood samples, indicating that human brain tissue is not frequently used for proteomics analyses in multiple sclerosis (Sandi et al., 2022). In the limited studies of human brain tissues conducted, several candidate proteins and pathways were found to be associated with the pathogenesis of multiple sclerosis. Using a combination of LCM and proteomics approaches, Han et al. identified proteins unique

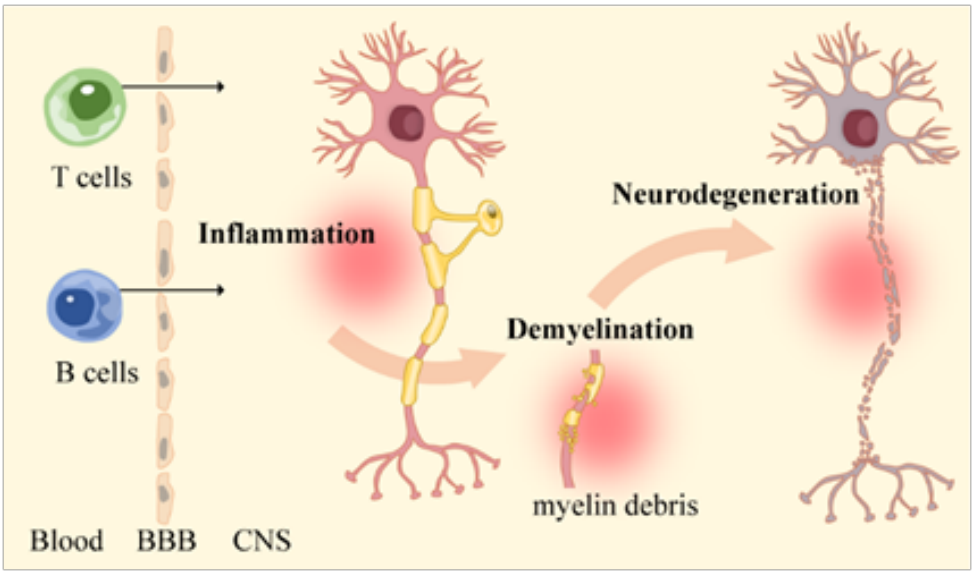

Figure 4. The pathological processes of multiple sclerosis.

to three major types of multiple sclerosis lesions (acute plaque, chronic active plaque and chronic plaque) in brain autopsy samples and revealed the extensive interface between the coagulation system and brain inflammation (Han et al., 2008). Moreover, the potential therapeutic role of protein C inhibitor, identified as a candidate protein in this study, was validated in experimental autoimmune encephalomyelitis (EAE, a classical animal model of multiple sclerosis) mice, emphasizing how lesion-specific proteomic profiling of diseased brain tissue from patients can be used to identify potential therapeutic targets. In 2011, Broadwater et al. characterized the mitochondrial proteome in postmortem multiple sclerosis cortex using surface-enhanced laser desorption ionization-time of flight mass spectrometry (SELDI-TOF-MS) and DE proteins involved in respiration were identified, including cytochrome C oxidase subunit 5b (COX5b), creatinine kinase B and hemoglobin-β (Broadwater et al., 2011). Furthermore, Ninjurin-1 was weakly expressed in healthy human brains but upregulated in active multiple sclerosis lesions, and Ninjurin-1 blockade reduced clinical disease activity in EAE mice (Ifergan et al., 2011). In another proteomics study, Chen and coworkers found that the levels of -associated proteins (including myelin basic protein, myelin proteolipid protein and myelin associated glycoprotein) significantly decreased in multiple sclerosis substantia nigra (Chen, Lu, Seeman, & Liu, 2012). In a MS-based study coupled with LCM, high levels of myelin proteins (most likely associated with extracellular myelin debris) were identified in demyelinated lesions from human multiple sclerosis brain, indicating the incomplete phagocytic clearance of myelin debris in multiple sclerosis lesions (Syed et al., 2016). A subsequent LC-MS/MS analysis of postmortem multiple sclerosis cortical tissues revealed proteins interacting with hemoglobin (Hb), including ATP synthase, histones, and histone lysine demethylase (Brown et al., 2016). In another study, Maccarrone and colleagues used MALDI-IMS to profile and investigate proteins/peptides expressed in human multiple sclerosis brain lesions with different degrees of remyelination (Maccarrone et al., 2017). They found that the molecular weight of lesions with low remyelination (<5,300 Daltons) was much lower than that of the lesions with more complete remyelination (>15,200 Daltons). Their results also suggested that thymosin-β4, a small molecular weight protein widely expressed in mammalian tissues has been shown to play a role in cell migration, inhibiting inflammation, and regulating remyelination process (Huff, Müller, Otto, Netzker, & Hannappel, 2001; Santra et al., 2012; Zhang et al., 2009), plays a neuroprotective role in the remyelination process occurring in the CNS of multiple sclerosis patients. Additionally, seven unique mutations of PLP1 associated with multiple sclerosis were identified in proteomics analysis, revealing a possible mutation-driven

mechanism underlies multiple sclerosis (Qendro et al., 2017). In 2019, Nicaise et al. evaluated brain tissue from deceased progressive multiple sclerosis patients and found that cellular senescence contributes to remyelination failure in progressive multiple sclerosis (Nicaise et al., 2019). Meanwhile, several citrullinated proteins, including MBP, vimentin, CN37, ermin, DPYL2 and NFM were detected in the multiple sclerosis brain (Faigle et al., 2019). Furthermore, a recent proteomic study indicated the activation of the MAPK and STAT pathways in the immune cells of multiple sclerosis patients, mainly in B cells (Kotelnikova et al., 2019).

All in all, the current human brain proteomics research highlights the importance of immune cells and chronic inflammation in the CNS of multiple sclerosis patients. Furthermore, reduced levels of myelin-associated proteins have been shown in multiple sclerosis brain tissues, confirming that oligodendrocyte degeneration and demyelination occur during this disease process. Potential therapeutic targets in multiple sclerosis (such as protein C, Ninjurin-1, and thymosin-β4 (Han et al., 2008; Ifergan et al., 2011; Maccarrone et al., 2017)) have also been identified through proteomics technologies. In addition to these advances, several studies have indicated the importance of other pathways, such as mitochondrial function, the coagulation system, phagocytosis, gliosis, glial and neuronal differentiation, in the development of multiple sclerosis (Broadwater et al., 2011; Han et al., 2008; Sandi et al., 2022; Syed et al., 2016). Thus, further studies are required to complete the picture.

Immune cells (T and B cells) enter the CNS through the disrupted blood-brain barrier (BBB), initiating inflammatory responses. Subsequently, oligodendrocytes are compromised, and demyelination occurs, leading to irreversible axon loss and neurodegeneration.

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a fatal adult-onset neurodegenerative disorder which limits survival to only 2–5 years after disease onset (Masrori & Van Damme, 2020). It is characterized by the progressive loss of motor neurons in the motor cortex, brainstem, and spinal cord (G. Kim, Gautier, Tassoni-Tsuchida, Ma, & Gitler, 2020; Mejzini et al., 2019; Taylor, Brown, & Cleveland, 2016; van Es et al., 2017). Aberrant protein aggregation and the formation of protein inclusions are hallmarks of ALS (Hedl et al., 2019). Several pathological proteins have been implicated in this disease, including TAR DNA binding protein 43 (TDP-43), superoxide dismutase 1 (SOD1), and tau (Hedl et al., 2019). ALS occurs with an incidence of approximately 1.75 per 100,000 people worldwide (De Marchi et al., 2019). Up to 10% of ALS cases are hereditary and defined as familial ALS (fALS), while the remaining 90%–95% of patients without a family history are considered sporadic ALS (sALS) (G. Kim et al., 2020; Mejzini et al., 2019). Studies have shown that *ALS's most common mutated genes are C9orf72, SOD1, TARDBP and FU*S (Zou et al., 2017).

Proteomic approaches have been used to explore the pathogenesis of ALS. In 2017, Engelen–Lee and colleagues conducted a fluorescent 2D-gel proteomic analysis combined with MS of postmortem spinal cord samples from individuals with ALS and controls (Engelen-Lee et al., 2017). Functional classification revealed that dysregulated proteins identified in the disease are involved in mitochondrial function (oxidative phosphorylation), intracellular calcium homeostasis, protein metabolism, glutathione homeostasis, protein transport and snRNP assembly. In 2018, Umoh and colleagues performed MS-based proteomics analysis of frontal cortical tissues from ALS cases carrying the C9orf72 mutation (a major genetic cause of ALS (DeJesus-Hernandez et al., 2011; Renton et al., 2011)) and sporadic ALS cases(Umoh et al., 2018). According to the weighted co-expression network analysis (WGCNA), C9orf72 expansion-positive ALS patients showed significantly increased levels of a module associated with astrocytic and microglia proteins compared to those in sporadic ALS cases, suggesting that the C9orf72 mutation is associated with neuroinflammation in the ALS brain. *SOD1* was the first risk gene described for ALS (Rosen et al., 1993). Over 200 *SOD1* variants have been described in patients with fALS (http://alsod.iop.kcl.ac.uk). In a recent study, Trist et al. revealed that structurally-disordered and immature SOD1 conformers mislocated and accumulated in spinal cord motor neurons of *SOD1*-linked and non-*SOD1*-linked ALS patients when compared with

controls, indicating that SOD1 without genetic alterations may contribute to motor neuron death in ALS patients by promoting pathways of SOD1 proteinopathy (Trist et al., 2022). Deposition of the hyperphosphorylated and ubiquitinated TDP-43 is the main pathology in affected neurons of patients with ALS and frontotemporal lobar degeneration (FTLD) (Prasad, Bharathi, Sivalingam, Girdhar, & Patel, 2019). In 2018, Iridoy and coworkers conducted a deep proteomic analysis of the spinal cord and frontal cortex from TDP-43 inclusion-positive ALS subjects, ubiquitin-positive FTLD subjects and controls without the neurodegenerative disease (Iridoy et al., 2018). In this study, 281 proteins were dysregulated in ALS cases compared to controls. Moreover, dysregulated protein interactome maps generated using the Ingenuity Pathway Analysis (IPA) tool implicated mitochondrial dysfunction and metabolic impairment in the process of ALS. In addition, protein interactions related to nucleic acid metabolism and energy production were over-represented in ALS. In 2019, Laferrière and colleagues developed the novel SarkoSpin method to separate pathological TDP-43 from the majority of normal proteins in human ALS and FTLD brain samples (Laferrière et al., 2019). By coupling SarkoSpin with MS, they found that phosphorylated TDP-43, extracted with numerous other abnormally insoluble proteins, forms distinct densities, polyubiquitination patterns, and morphologies associated with disease subtypes. This finding is consistent with the view that disease heterogeneity originates from alternate pathological TDP-43 conformations. Another proteomics study identified several novel phosphorylations, deamidation, and cleavage sites in pathological TDP-43 prepared from ALS-diseased human brains and showed that almost all modifications were localized in the glycine-rich C-terminal half, providing evidence that TDP-43 modification can affect the molecular pathways of this disease (Kametani et al., 2016). Liu and coworkers identified differentially expressed GFAP clusters in postmortem ALS and non-ALS spinal cords by 2D SDS-PAGE (Liu et al., 2013). Additionally, using LC-MS/MS, they revealed that the acetylated larger forms of GFAP fragments are upregulated in ALS spinal cord, indicating that dysregulation of protein acetylation may be involved in the pathogenesis of ALS. Alonso et al. demonstrated the occurrence of several fungal peptides in the frontal cortex in ALS patients, suggesting that fungal infection may also play a role in the pathology of ALS (Alonso et al., 2015). Moreover, in recent proteomic profiling of motor cortex extracellular vesicles (MCEVs) isolated from motor cortex brain tissues of ALS patients and matched controls, a panel of statistically significant DE proteins were identified in ALS MCEWs compared to control MCEWs (Vassileff et al., 2020). This information could be used to develop a panel of extracellular vesicle biomarkers for detection in CSF and blood samples. Furthermore, this panel included two upregulated RNA-binding stress granule-associated proteins (i.e., STAU1 and DHX30), substantiating the connection between ALS and stress granules and highlighting the potential roles of small extracellular vesicles in the pathogenesis of ALS.

Collectively, human brain proteomics analyses have implicated many cellular and molecular processes in ALS, including mitochondrial dysfunction, aberrant protein aggregation, neuroinflammation, metabolic impairment and impaired protein degradation (**Fig. 5**). However, although multiple mechanisms appear to be proposed, the cytoplasmic aggregation of aberrant TDP-43 is a pathological hallmark in most cases of ALS. It plays a key role in driving neurodegeneration (Mejzini et al., 2019). TDP-43, encoded by *TARDBP*, is a DNA/RNA-binding protein generally localized to the nucleus and plays a role in RNA processing and metabolism, including mRNA splicing, stability, transport and translation (Deshaies et al., 2018; G. Kim et al., 2020; Neelagandan et al., 2019). However, abnormal TDP-43 aggregates in the cytosol, where it acquires toxic properties and/or leads to a loss of function in the nucleus (Mejzini et al., 2019). Additionally, several PTMs, like ubiquitination, phosphorylation and acetylation, are associated with TDP-43 proteinopathy (Buratti, 2018; Laferrière et al., 2019; Liu et al., 2013). Despite these advances, extensive and in-depth analysis is still needed to fully elucidate the pathophysiological mechanisms underlying the formation of TDP-43 aggregation and its role in the pathogenesis of ALS.

Several pathways have been implicated in the pathogenesis of ALS, including mitochondrial dysfunction, aberrant protein aggregation, neuroinflammation, metabolic impairment and

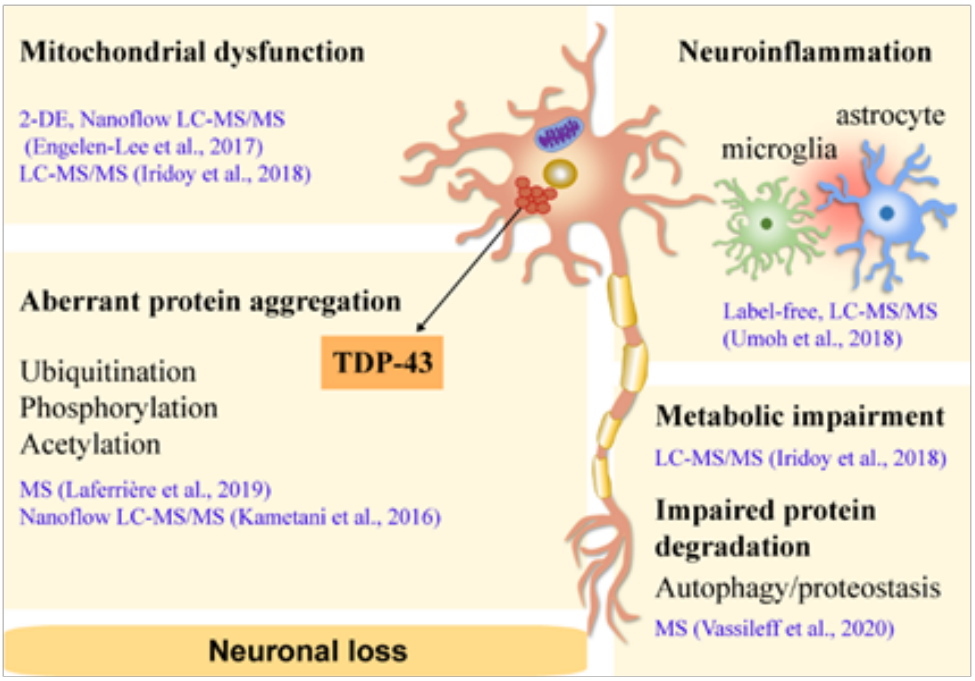

Figure 5. Pathogenic pathways implicated in ALS according to human brain proteomics.

impaired protein degradation. The methods used for analysis are shown in blue.

Huntington's disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by the abnormal expression of mutant huntingtin (mHTT) protein containing an expansion of a glutamine stretch in its N-terminal sequence. Clinically, it is characterized by progressive extra-pyramidal motor dysfunction, psychiatric disturbance and cognitive decline, eventually leading to death (Lontay, Kiss, Virág, & Tar, 2020; Ross, Pantelyat, Kogan, & Brandt, 2014). HTT is ubiquitously expressed and extensively involved in CNS development, axonal transport, synaptic function and cell survival (Saudou & Humbert, 2016; Tabrizi, Flower, Ross, & Wild, 2020). Evidence suggests that mHTT affects many cellular functions, finally leading to cell death. HD neurodegeneration is particularly severe in the corpus striatum (caudate nucleus and putamen) (Rüb et al., 2016). However, neuronal dysfunction and death also occur in the cerebral cortex (E. B. Johnson et al., 2021). A wide variety of PTMs are thought to occur within HTT (e.g., phosphorylation, sumoylation, acetylation, palmitoylation, ubiquitination, and protease cleavage), which regulate its stability, aggregation, subcellular localization and clearance (Ehrnhoefer, Sutton, & Hayden, 2011; Saudou & Humbert, 2016).

Previous proteomics studies of HD, which have mainly been carried out with samples from mouse and cell models, have identified numerous cellular processes and pathways associated with HD, including energy metabolism, gene transcription, protein translation, RNA processing, cytoskeleton dynamic, and protein trafficking (Chiang et al., 2007; Culver et al., 2012; Ratovitski et al., 2012; Shirasaki et al., 2012; Wegrzynowicz, Holt, Friedman, & Bowman, 2012; Zabel et al., 2009). However, only a few proteomics studies have been conducted on human HD brain tissues (Ratovitski et al., 2016). DiProspero and associates reported a dramatic, progressive loss of neurofilament protein throughout the cortical tissue of HD patients, which could influence axonal diameter, alter conduction velocities, and participate in the pathogenesis of HD (DiProspero et al., 2004). In 2008, Sorolla et al. combined 2D gel electrophoresis with MALDI-TOF to analyze the proteome of striatum and cortex of HD affected human brain and control samples. They provided further evidence of the critical role of oxidative stress in HD pathology (Sorolla et al., 2008).

Additionally, most DE proteins were observed in both striatum and cortex. Among these proteins, GFAP and αB-crystallin were highly upregulated in HD samples, indicating the occurrence of reactive gliosis in HD. Chen et al. utilized nano-UPLC and MS-based label-free quantitative proteomics to investigate the protein alterations in the substantia nigra of HD patients (Chen et al., 2012). They identified 22 proteins with significant changes in expression levels, consisting of five upregulated proteins (dynein light chain, histone H2B, enolase, brain acid soluble protein 1 and guanine nucleotide-binding protein beta subunit 4) and 17 downregulated proteins. These findings emphasized the functional adaption or damage in the substantia nigra in HD. Interestingly, six of the downregulated proteins are associated with energy metabolism (amine oxidase B, ATP synthase coupling factor 6, creatine kinase B, cytochrome c oxidase subunit 6B1, glucose-6-phosphate 1-dehydrogenase and NADH dehydrogenase iron-sulfur protein 3), which is in accordance with previous findings indicating that energy metabolism defects play essential roles in the pathogenesis of HD (Walker & Raymond, 2004). Additionally, by using 2D gel electrophoresis and MALDI-MS in a proteomics study of middle frontal gyrus (MFG) and visual cortex (VC) samples from postmortem HD brain tissues and matched controls, Schönberger et al. identified 22 DE proteins in the MFG and only seven in the VC (Schönberger, Jezdic, Faull, & Cooper, 2013). The DE proteins identified implied functional impairment of cellular processes in the progression of HD, including increased stress response, activation of apoptosis, impaired glycolysis, disrupted vesicular trafficking/endocytosis, impaired axonal function and ubiquitin-proteasome system (UPS) dysfunction. Recently, the iTRAQ-based LC-MS/MS approach was used to quantify the changes in protein abundances in the superior frontal gyrus of HD and control cases (Ratovitski et al., 2016). In total, 4,789 proteins were quantified, with significant changes detected in the expression of 1,211 proteins in HD cases compared and control cases. IPA revealed that DE proteins were enriched in several cellular pathways, including Rho-mediated signaling, actin cytoskeleton dynamics, integrin signaling, mitochondrial dysfunction, endocytosis, protein folding, axonal guidance, DNA/RNA processing and protein transport, which partially confirm previously identified pathogenic pathways in HD (Consortium, 2012; Duan, Jiang, & Jin, 2014; Lin & Beal, 2006; Ross, Aylward, et al., 2014; Ross & Tabrizi, 2011).

On the whole, accumulating evidence demonstrates that energy metabolism disruption and mitochondrial dysfunction play essential roles in the pathogenesis of HD. However, other pathways have also been identified, such as Rho-mediated signaling, actin cytoskeleton regulation, axonal guidance and DNA/RNA processing. In short, the expansion of CAG repeats in the huntingtin gene (*HTT*) causes misfolding and aggregation of mHTT, which leads to mitochondrial dysfunction and energy metabolism defects in HD, with the involvement of increased lactate concentration, decreased glucose metabolism and impaired ATP production (Illarioshkin, Klyushnikov, Vigont, Seliverstov, & Kaznacheyeva, 2018; W. R. Martin, Wieler, & Hanstock, 2007; Milakovic & Johnson, 2005). Furthermore, protein degradation systems are compromised in the pathogenesis of HD, including the decreased function of the ubiquitin-proteasome system (UPS, the main route for degradation of abnormally folded proteins in mammalian cells) and disruption in autophagy (Bennett et al., 2007; Cortes & La Spada, 2014). In turn, inhibited UPS and impaired autophagy increase mHTT misfolding and aggregation, which impede mHTT clearance (**Fig. 6**).

The expansion of CAG repeats leads to misfolding and aggregation of mHTT in the neurons, which causes mitochondrial dysfunction and energy metabolism disruption and also influences cellular pathways such as Rho-mediated signaling, actin cytoskeleton regulation, axonal guidance, DNA/RNA processing, protein transport, oxidative stress, gliosis and activation of apoptosis. Additionally, mHTT clearance pathways are impaired (including decreased UPS function and autophagy), increasing protein misfolding and aggregation. The methods used for analysis are shown in blue.

Limitations and future perspectives

Quantitative proteomics analyses of diseased brain tissues directly address the changes in protein abundance that occur during the progression of neurodegenerative diseases, thus potentially providing insights into the disease mechanisms and identifying potential biomarkers. Undoubtedly, human brain samples would give the most direct

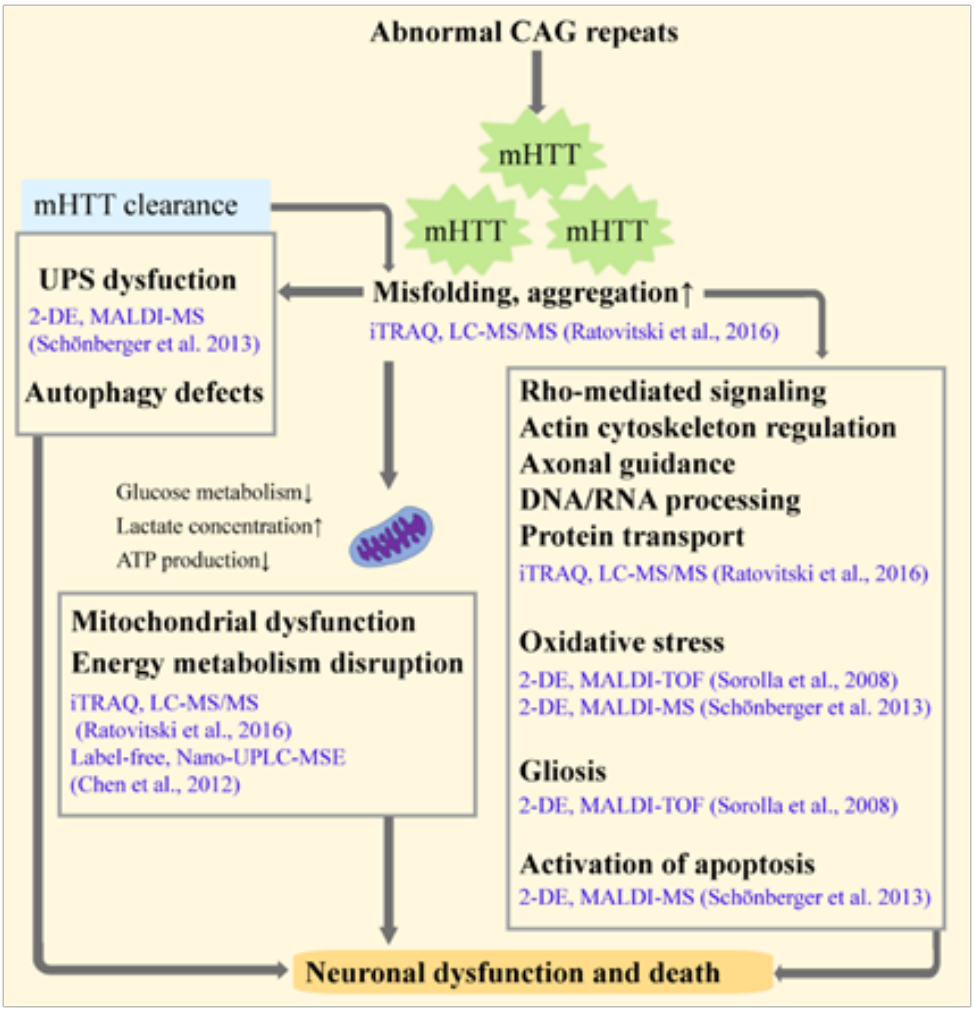

Figure 6. Pathways involved in the pathogenesis of HD according to human brain proteomics.

source for analyzing neurodegenerative diseases; however, due to problems in accessibility and ethical concerns (Ward, Güntert, Campbell, & Pike, 2009), the availability of human brain tissues is restricted to autopsies and rare biopsies. To date, a plethora of data has been generated in research conducted on human fluid samples (e.g., CSF, blood, saliva, tears, and urine), with all the associated limitations. Future efforts are required to optimize the extraction method of human brain tissue and further improve the efficiency with which the tissue obtained is utilized. Advances in this field are also limited by the relative lack of region-specific and cell-specific proteomics studies based on diseased human brain tissues. Fractionation of the desired tissues or cells is crucial, using processes that require sophisticated and elaborate techniques. Future studies should combine highly sensitive techniques for sub-proteome analysis of human brain tissue and further explore disease mechanisms related to specific regions or cell types.

Single-cell proteomics is the recently developed mass cytometry technique. More than 1000 proteins can be detected by a single-cell proteomics (Perkel, 2021). Although limitation exists, single-cell proteomics can offer cell-type-specific signaling responses in complex and heterogeneous organs, such as brains (He, Memczak, Qu, Belmonte, & Liu, 2020). Separation of entire cells, such as neurons, from postmortem human brains, is difficult, which limits the development of single-cell proteomics in human brains. Further studies could develop new single-cell separation methods from postmortem human brains, and new

techniques to simultaneous identify and quantify more peptides in a single cell.

Conclusion

Proteomics studies have indicated a broad, dynamic proteomic perturbation during the progression of neurodegenerative diseases. Some shared features of neurodegenerative diseases have been mentioned in human brain proteomics studies (e.g., abnormal protein accumulation and mitochondrial dysfunction). Combining bioinformatics and big data analytics, advances in the human brain proteomics offered a new vision of neurodegenerative disease mechanisms that can be used to identify and develop novel biomarkers and therapeutic targets. The aim of this review was not to present a detailed description of proteomics research related to various neurodegenerative diseases, but rather to provide an overview of human brain proteomics studies related to the most prevalent neurodegenerative disorders (AD, PD, multiple sclerosis, ALS and HD) and stimulate further exploration of these diseases.

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Conflict of Interest Disclosures

The authors have declared that no conflict of interest exists.

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