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*Review*

# Post-translational modifications in neurodegeneration

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**Abstract:** Post-translational modifications increase proteome functionality for managing all aspects of normal cell biology. They are based on the covalent attachment of functional groups, leading to phosphorylation, acetylation, glycosylation, acylation, ubiquitination, SUMOylation and oxidation of protein targets. Post-translational modifications occur at any step of protein life cycle, modulating in time and space protein folding, subcellular localization and activity. Aberrant post-translational modifications of one or more culprit proteins may lead to neurodegeneration, as shown in paradigmatic neurological disorders such as Alzheimer's, Parkinson's and prion diseases. In this review, we report the most important post-translational modifications found in neurodegenerative disorders, illustrating the pathophysiological mechanisms in which they are involved. This work highlights the lack of a global framework of post-translational modifications in terms of complexity and regulation. Therefore, in the next future many efforts are required to describe the interplay existing between post-translational modifications and their combinatorial patterns on protein targets.

**Keywords**: post-translational modifications; neurodegeneration; phosphorylation; acetylation; glycosylation; acylation; ubiquitination; SUMOylation; deamidation; oxidation

## 1. Introduction

Since the first draft of human genome was complete, it has been immediately clear that the estimated number of protein-coding genes—between 20,000 and 25,000—was not sufficient to account for the complexity of biological functions shown by eukaryotic cells [1]. To escape this "genetic prison", evolution has shaped a series of molecular mechanisms in order to increase the

coding capacity of the genome of several orders of magnitude, allowing organisms to better adapt and face the challenges of a changing environment.

At transcriptional level, alternative splicing and differential promoters/terminators usage can significantly increase the number of messenger RNAs encoded by a single gene, bringing the human transcriptome to over 100,000 potential transcripts [2]. In addition, covalent modifications to specific amino acids raise the human proteome to one million proteins [3]. Modifications occurring at target amino acids are collectively known as post-translational modifications (PTMs), independently by their biochemical nature. Unlike genetic variations which take place on an evolutionary time scale, PTMs act as molecular switches in response to cellular and extra-cellular stimuli. Multiple PTMs can simultaneously occur and cooperate to dictate the molecular state of a protein including its conformation, enzymatic activity, cellular localization, turn-over and interactions with other biomolecules. To date, more than 90,000 individual PTMs have been identified through biochemical and biophysical analyses [4].

Considering the crucial importance of PTMs for protein function, they are strictly regulated in space and time. As a consequence, any dysregulation of PTM homeostasis can potentially lead to a pathological state. Among others, a number of neurodegenerative diseases are characterized by aberrant PTMs of one or more culprit proteins. In this review, we present an overview on the principal PTMs with an emphasis on their regulation in health and disease. In particular, we reported the pathophysiological mechanisms in which PTMs are involved and how they determine neuronal loss in a few paradigmatic neurological disorders.

## 2. Phosphorylation

Protein phosphorylation is the most widespread PTM. Virtually all types of extracellular signals exert their physiological effects by modulating phosphorylation of specific proteins in target cells. Phosphorylation is a reversible modification principally occurring at serine residues of protein substrate (95%), and to a lesser extent on threonine (4%) and tyrosine (1%) residues [5]. The process is mediated by protein kinases that catalyze the transfer of the γ-phosphate group of ATP to the hydroxyl group of target residues. Their action is counterbalanced by protein phosphatases, working antagonistically for promoting the hydrolysis of newly formed phosphoester bonds [6]. Thus, the final phosphorylation pattern is the result of the concerted activity of these two classes of enzymes. An imbalance in this equilibrium hence produces the hyper- or hypo-phosphorylation of target proteins. Hereafter we discuss two examples in which aberrant phosphorylation plays a pivotal role in pathogenesis of neurological disorders.

### *2.1. Tau phosphorylation in tauopathies*

Tauopathies belong to a class of neurodegenerative disorders encompassing more than 20 clinicopathological entities, including Alzheimer's disease (AD), progressive supranuclear palsy (PSP), Pick's disease (PiD), corticobasal degeneration and post-encephalitic Parkinsonism. At the molecular level, they are characterized by the presence in neurons of insoluble aggregates called neurofibrillary tangles (NFTs), mainly constituted of misfolded tau protein organized in paired helical filaments (PHFs) [7].

Tau is a naturally unfolded protein highly expressed in the central nervous system (CNS), and existing in 6 isoforms due to alternative splicing of exons 2, 3 and 10 in the *MAPT* gene. Each isoform contains a different number of N-terminal inserts (0N, 1N or 2N) and C-terminal microtubule-binding repeats (3R or 4R) [8]. In mature neurons, tau is localized mainly in the axons where it promotes microtubule assembly by binding their surfaces laterally [9]. The longest isoform (2N4R) contains 85 potential phosphorylation sites located within the C-terminal repeats and the flanking regions. In physiological conditions approximately 30 of them are phosphorylated through the activity of different kinases such as glycogen synthase kinase (GSK)-3β, cyclin-dependent kinase 5 (CDK5) and the MAPK family [10]. Upon disease, aberrant hyper-phosphorylation takes place at specific "pathological sites". The causes of this abnormal process are not fully elucidated yet. Initial evidence pinpointing at up-regulation of CDK5 in AD brain has been recently questioned [11]. One of the most robust candidates is currently protein phosphatase 2A (PP2A) whose activity seems to be inhibited upon AD due to up-regulation of the endogenous inhibitors $I_1^{PP2A}$ and $I_2^{PP2A}$ [12]. Consistently with this hypothesis, other PPA2 substrates are hyper-phosphorylated in AD brain as well [13,14].

Tau hyper-phosphorylation is considered a key event in triggering tau aggregation since it precedes NFTs formation. While tau binds microtubules more efficiently in its less phosphorylated form [15], hyper-phosphorylation could induce tau detachment from microtubule network with subsequent self-organization into high ordered structures such as PHFs. In addition, phosphorylation of certain epitopes may directly influence local tau conformation, making it more prone to assembly into PHFs. For instance, the phosphorylation state of the most prominent AD pathological sites—the epitopes recognized by antibodies AT8 (pS199/pS202/pT205), AT100 (pT212/pS214), and PHF-1 (pS396/pS404)—has been shown to induce a compaction of the paperclip folding of tau inducing a pathological conformation [16]. The molecular mechanisms underlying NFTs neurotoxicity are not completely understood. It has been proposed that tau aggregates affect neuronal survival through a toxic gain-of-function by sequestering important cellular factors. In recent years, soluble tau oligomers have emerged as the real toxic species while larger aggregates could instead be neuroprotective for removing oligomers from the cytosol and inactivate them [17].

Disruption of physiological patterns of tau phosphorylation can determine neuronal loss independently from aggregation. Tau hyper-phosphorylation affects normal cytoskeletal architecture and microtubule dynamics, resulting in axonal transport blockage [18]. Abnormal phosphorylation also alters tau subcellular distribution, promoting its relocation from the axon to the somato-dendritic compartment [19]. The accumulation of hyper-phosphorylated tau within dendritic spines was shown to alter synaptic function by impairing glutamate receptor trafficking or synaptic anchoring [20]. Furthermore, the evidence that aggregated tau is also ubiquitinated suggests that tau hyper-phosphorylation may affect its turn-over through the failure of the ubiquitin-proteasome system (UPS) pathway [21].

### *2.2. α-synuclein phosphorylation in Parkinson's disease*

Parkinson's disease (PD) is a progressive neurological disorder characterized by loss of dopaminergic neurons in substantia nigra *pars compacta*. The selective neurodegenerative process leads to a broad range of clinical symptoms including bradykinesia, rigidity, loss of postural reflex

and resting tremor. At a cellular level, dying dopaminergic neurons show intracytoplasmatic inclusions of α-synuclein, known as Lewy bodies (LBs) [22].

α-synuclein is a natively unfolded protein highly enriched in the presynaptic terminals of neurons in the CNS. Its physiological function is poorly understood, though several lines of evidence suggest a role in neurotransmitter release [23]. The primary structure contains 5 phosphorylation sites (Ser87, Ser129, Tyr125, Tyr133, and Tyr136) that can be phosphorylated by different kinases such as G-protein coupled receptor kinases (GRKs), casein kinases 1 and 2 (CK1 and CK2), and the polo-like kinases (PLKs) [24]. In physiological conditions approximately 4% of α-synuclein is phosphorylated at the level of Ser129, whist in LBs the amount of the phosphorylated form reaches 90% [25]. Phospho-Ser87 is also enhanced in PD and related disorders, while Tyr125 shows lower phosphorylation content in cortical tissue from PD patients than healthy controls [26,27]. These findings strongly support an involvement of α-synuclein phosphorylation in PD etiology, though its role in LBs formation is still elusive. Contrasting results on the role of Ser87 and Ser129 phosphorylation in inducing α-synuclein aggregation are available [28,29,30]. Discrepancies might be due to the different experimental systems used [31]. Increased oxidative stress or proteasomal inhibition has been proposed to explain α-synuclein hyper-phosphorylation [32]. Alternatively, α-synuclein aggregation might be the cause for its hyper-phosphorylation as aggregates could be accessible to protein kinases but not to phosphatases [33]. Similar to tauopathies, the contribution of LBs to neuronal loss is debated and α-synuclein oligomers rather than larger aggregates seem the molecular species driving neurodegeneration.

Besides the neurotoxic effects of aggregates, evidences on the cytotoxic role of α-synuclein phosphorylation have been collected. Ser129 phosphorylation was demonstrated to modulate the shuttling of α-synuclein between cytosol and nuclear compartments [34]. Once in the nucleus, α-synuclein is able to interact directly with histones and mask them from the action of histone deacetylases (HDACs), eventually inducing neurotoxicity [35]. Phosphorylation at Ser129 and Ser87 might also contribute to abnormal synaptic transmission in PD since they reduce α-synuclein binding to lipid vesicles, possibly impairing dopamine release [26,36]. Phosphorylation of Ser129 and Tyr125 were also shown to shift α-synuclein interactome from a prevalence of mitochondrial transporters to proteins involved in vesicular trafficking and serine phosphorylation, suggesting dysfunctions in energy production in PD phenotype [37].

## 3. Acetylation

Acetylation is one of the most common PTMs and consists in the reversible transfer of an acetyl group from the acetyl-coenzyme A to the ε-amino group of the side chain of lysine residues. Two classes of enzymes catalyze this PTM: histone acetyl transferases (HATs) and histone de-acetylases (HDACs). Their names derive from their best known substrates, though an increasing number of non-histone proteins have been recently found subjected to acetylation in global proteomic screening, implying a much broader activity for these enzymes in cell biology [38]. In neurodegeneration, several pathological venues are caused by an imbalance in the delicate HAT/HDAC equilibrium of the nervous system. In this section, the effects of aberrant acetylation on histone and non-histone substrates will be presented.

### *3.1. Histone proteins*

Histone acetylation is one of the best-understood mechanisms and the most prominent epigenetic mark in the CNS [39]. This PTM reduces the overall positive charge of histones and promote an open chromatin conformation by decreasing the interaction with the negatively charged phosphate groups of the DNA. This change in chromatin topology allows the transcription of genes in that portion of the genome. Moreover, the acetyl tags themselves represent functional docking sites for transcriptional activators and epigenetic "readers" [40]. In physiological conditions, the antagonistic activity of HATs and HDACs determines the net acetylation state of the chromatin, directly controlling gene expression. Upon disease, aberrancies in histone acetylation have been connected to the etiology of several neurodegenerative disorders in two possible ways. In the first modality, hypo-acetylation at a specific genetic locus decreases the expression of a protein causing disease by a loss-of-function mechanism. In the second one, histone hypo-acetylation takes place at multiple genetic loci and causes wide-spread transcriptional deficits.

A prime example of the first scenario is Friedreich ataxia. This recessive ataxia is caused by a pathogenic expansion of GAA triplet repeats located in the first intron of the frataxin (*FXN*) gene [41]. The expanded repeat induces gene silencing through a process of heterochromatinization partially mediated by hypo-acetylation of specific lysine residues on histones around the trinucleotide repeats and on the promoter. They include Lys9 and Lys14 on histone H3 and Lys8, Lys12 and Lys16 on histone H4 [42,43]. Fragile X syndrome is a neurological disorder caused by silencing of *FMR1* gene through an analogous mechanism. In this pathology, hypo-acetylation is due to a CGG trinucleotide expansion located in the 5'-UTR of the gene [44].

An excellent example of the second scenario is given by polyglutamine (polyQ) diseases. They are a group of 9 neurodegenerative disorders characterized by the expansion of a CAG repeat encoding for a polyQ tract in the wild-type protein. Although different proteins are involved—huntingtin (HTT) in Huntington's disease (HD); ataxins (ATXNs) in spinocerebellar ataxias (SCAs) 1, 2, 3, 6, 7; TATA-binding protein (TBP) in SCA17; androgen receptor in spinal bulbar muscular atrophy (SBMA) and atrophin-1 (ATN1) in dentatorubropallidoluysian atrophy (DRPLA)—they all act as transcription factors [45]. The polyQ expansion is believed to alter global chromatin acetylation and induce transcription defects through different mechanisms. PolyQ proteins can repress transcription by directly inhibiting HAT activity, as the case of HTT mutant able to bind and sequester the HAT CREB-binding protein (CBP) from the soluble pool of nuclear factors [46]. Other polyQ proteins (such as ATXN3) were shown to silence transcription by binding to histones and blocking acetylation sites [47]. Finally, polyQ proteins like ATXN1 decrease histone acetylation by recruiting HDACs at the level of promoter regions of target genes [48,49].

### *3.2. Non-histone proteins*

The first cytosolic substrate of the HAT/HDAC system to be discovered was α-tubulin. In physiological conditions, α-tubulin is acetylated at the level of a conserved residue (Lys4) which is postulated to reside on the luminal face of microtubules [50]. The specific HAT involved in this process is still unknown, while two HDACs—HDAC6 and SIRT2—have been found to deacetylate α-tubulin both *in vitro* and *in vivo* [51,52]. Tubulin hyper-acetylation via inhibition of HDAC enhances axonal trafficking of mitochondria in primary hippocampal neurons [53], supporting the

hypothesis that alterations in α-tubulin acetylation may play a role in neurodegenerative disorders with impaired axonal transport such as AD, HD and Charcot-Marie-Tooth disorders (CMT). Consistently with this model, reduced levels of acetylated α-tubulin have been detected in AD and HD brains [54,55]. Recently, it has been shown that pharmacological inhibition of HDAC6 rescues axonal trafficking and restores tubulin acetylation in a CTM mouse model characterized by axonal transport defects [56].

Tau represents another cytosolic HAT substrate with relevance in pathology since acetylated tau was detected exclusively in brain lesions of AD and other tauoapthies. In particular, Lys280 in the microtubule-binding motif was identified as the principal target site for the concerted activity of HAT p300 and HDAC SIRT1 [57]. More recently, Lys174 as well has been characterized as a pathological acetylation site in the early stages of disease [58]. Similar to phosphorylation, acetylation has the potential to reduce tau affinity for microtubules promoting its pathological aggregation [59]. Tau acetylation seems to be the cause of phosphorylated tau accumulation as consequence of the failure of its proteolytic degradation [57]. In the future, it will be important to understand the interplay between the different PTMs for tau pathophysiology.

## 4. Glycosylation

Glycosylation is one of the most complex PTMs, and occurs in more than half of the human proteome [60]. It takes place in the endoplasmic reticulum(ER)/Golgi compartment through an elaborate multi-step enzymatic reaction leading to the formation of a variety of protein-bound oligosaccharides with diverse biological functions. The three main PTMs involving carbohydrates are N-linked and O-linked glycosylation, and glypiation [61]. Remarkably, all these three types have been connected to neurodegeneration at different levels.

### *4.1. N-linked glycosylation*

N-linked glycosylation consists in the transfer of a precursor glycan from an isoprenoid lipid carrier to the side chain amide of selected asparagine residues. This reaction is catalyzed by oligosaccharyltransferase in the ER. These target residues are specified by the consensus sequence Asp-X-Ser/Thr. The core oligosaccharide is then remodeled by other glycosyltransferases and glycosidases in the Golgi apparatus, giving rise to three main types of glycans: high mannose, hybrid and complex glycans [62].

In the context of neurodegeneration, prion protein ($PrP^C$) is an excellent example of such glycosylation. $PrP^C$ is a cellular protein that is converted into a pathological conformer ($PrP^{Sc}$ or prion), causing the so-called transmissible spongiform encephalopathies (TSEs) or prion disorders [63]. In physiological conditions, $PrP^C$ exists as un-, mono- or di-glycosylated form due to the presence in its primary sequence of two asparagines (Asn181 and Asn187 in human $PrP^C$) that can undergo N-linked glycosylation. Although $PrP^C$ glycosylation is not necessary for conversion [64], glycosylation patterns of $PrP^{Sc}$ show strain-specific properties and may contribute to the molecular basis of TSE strain variation [65]. Indeed, differences in the glycoform ratio or composition have been observed among diverse prion diseases [66,67]. Additionally, the glycosylation state of $PrP^C$ was shown to influence the conformation of $PrP^{Sc}$-like molecules obtained in a cell-free system [68].

### *4.2. O-linked glycosylation*

Unlike the N-linked, O-linked glycosylation starts directly in the Golgi apparatus where an N-acetyl galactosaminyltransferase transfers an N-acetylgalactosamine (GalNAc) residue to the hydroxyl-oxygen in side chain of a serine or a threonine residue for which no specific consensus exists. Subsequently, the sugar moiety is further elongated or modified by sialylation, sulfatation, acetylation, fucosylation, and polylactosamine extension [69].

Remarkably, this type of glycosylation was shown to counteract phosphorylation at the same or neighboring sites on the protein backbone [70]. This ability might have profound implications for those neurodegenerative venues characterized by aberrant protein phosphorylation such as tau in AD [71]. This hypothesis is corroborated by the evidence that the tau-enriched cytoskeletal fraction from AD brains shows a significant decrease in O-GlcNAc glycosylation compared to controls [72]. Consistently, overexpression of O-GlcNAc transferase in cultured cells is sufficient to alter tau phosphorylation patterns, inducing the de-phosphorylation of several target sites including Ser202, Ser396 and Ser404 [72].

### *4.3. Glypiation*

In this type of glycosylation a pre-formed glycosylphosphatidylinositol (GPI) anchor is attached to a protein by the action of a GPI transamidase. This enzyme cleaves the peptide bond at the GPI-anchor attachment site—the omega-site, which is near the C-terminus of the protein—and creates an amide linkage between the ethanolamine of the GPI and the newly generated carboxyl group at the end of the cleaved precursor protein [73]. This moiety allows the attached protein to anchor to the outer leaflet of the cell membrane or within the lumen of intracellular organelles. GPI-anchored proteins perform a diverse set of functions including roles in signal transduction, cell adhesion and antigen presentation [74].

A paradigmatic example of this PTM is again $PrP^{C}$. Indeed, during the maturation process, $PrP^{C}$ is linked to a GPI anchor which targets the protein to specialized microdomains of the cellular membrane called “lipid rafts”, highly enriched in sphingolipids and cholesterol [75,76]. A few studies in cellular models of prion replication have highlighted the importance of $PrP^{C}$ localization in lipid rafts for its conversion to $PrP^{Sc}$, pinpointing a primary role for the GPI anchor in the pathogenic process [77,78]. In contrast, transgenic mice expressing anchorless $PrP^{C}$ were shown to be susceptible to prion infection as well as to accumulate $PrP^{Sc}$ [79]. Interestingly, these mice did not show any clinical sign associated with prion infection, suggesting that GPI anchor may be more important for mediating neurotoxic signals rather than for the conversion itself [79]. Furthermore, recent experimental evidence proposes that GPI anchor could also be necessary for the establishment of persistent prion infection [80].

## 5. Fatty Acylation

Fatty acylation consists in the covalent attachment of fatty acids to proteins. Initially described in viral proteins, this PTM has been found in a wide range of eukaryotic membrane glycoproteins having a pivotal role in cellular structure and function such as signal transduction and cell binding [81]. The two most common forms of protein fatty acylation are modification with palmitate,

a 16-carbon saturated fatty acid, and myristate, a 14-carbon saturated fatty acid [82]. Both types are dynamically modulated and their dysregulation has been recently found involved in several neurodegenerative processes.

### *5.1. Palmitoylation*

This type of fatty acylation consists in the reversible attachment of a palmitoyl group to the sulfhydryl group of a cysteine residue via a labile thioester bond, catalyzed by S-palmitoyl transferases (PATs) within the Golgi apparatus. No strict consensus sequence exists for the target cysteines but they are preferentially surrounded by basic or hydrophobic amino acids located in transmembrane domains or in the cytoplasmic flanking regions [83]. The opposite reaction is mediated by specific acyl protein thioesterases. Two enzymes—PPT1 and APT1—have been characterized in mammals but only APT1 appears to de-palmitoylate cytoplasmic proteins *in vivo* while PPT1 is mainly involved in their lysosomal degradation [84]. It has been shown that palmitoylation increases the association of a protein to the cell membrane [85].

In the nervous system, protein palmitoylation is particularly important for a number of processes including neuronal development and synaptic activity. Aberrant palmitoylation has been shown to contribute to several neurodegenerative diseases including HD and AD. PolyQ expansion in HTT was demonstrated to reduce the affinity of the protein for its specific PATs huntingtin interacting protein 14 (HIP14) and HIP14-like (DHHC13), resulting in reduced HTT palmitoylation at Cys214 which is essential for its trafficking and functions [86]. Consistently, palmitoylation-resistant HTT mutants exhibit increased neurotoxicity and propensity to form aggregates, supporting the role of hypo-palmitoylation in determining HD phenotype [86]. In contrast, a decrease in palmitoylation might be beneficial in the context of AD. Indeed, palmitoylation of amyloid precursor protein (APP) at Cys186 and Cys187 enhances its amyloidogenic processing by targeting APP to lipid rafts and enhancing its γ-secretase mediated cleavage [87]. Interestingly, the γ-secretase complex itself is also subjected to palmitoylation and its regulation might be functionally connected to Aβ-burden in the brain. In facts, the expression of a palmitoylation-deficient form of γ-secretase in an AD mouse model significantly reduced the amount of insoluble Aβ as well as amyloid deposition in the frontal cortex [88].

To sum up, the role of palmitoylation in neurodegeneration is not univocal as contrasting effects have been reported. In general, aberrant palmitoylation exerts its detrimental effects on neuronal survival mainly via perturbing the correct sub-cellular localization of specific target proteins.

### *5.2. Myristoylation*

This type of acylation encompasses the attachment of a myristoyl moiety to the α-amino group of an N-terminal glycine residue, via an amide bond. Unlike palmitoylation, this PTM is an irreversible process that takes place in the cytosol through the activity of the ubiquitous enzymes N-myristoyltransferases (NMTs) [89]. The specificity of these enzymes towards their substrate proteins is given by the consensus sequence Met-Gly-X-X-X-Ser/Thr. During translation, the initial Met is removed by a methionine amino peptidase, making the glycine in position 2 the N-terminal residue [82].

Recent evidence suggests that myristoylation may play a role in HD by modulating the autophagy pathway. Indeed, it has been shown that the internal Gly553 of HTT undergoes myristoylation following caspase cleavage at Asp552 and in turn promotes the formation of autophagosomes [90]. Importantly, this process was demonstrated to be reduced in mutant HTT compared to the wild type protein, maybe due to steric hindrance of the abnormal polyQ stretch or to NMT inhibition [91]. Thus, it is possible that the pathological polyQ expansion in HTT may alter the physiological autophagy dynamics via decreasing mysristoylation at Gly553, contributing to the build-up of cytotoxic materials observed upon HD [92].

## 6. Ubiquitination

Ubiquitination affects cellular processes inducing protein degradation [93], modulating cellular localization of proteins, activating and inactivating proteins, and mediating protein-protein interactions [94,95]. In particular, the UPS is the major proteolytic pathway used by eukaryotic cells for disposing of misfolded or damaged proteins [93]. Ubiquitination consists in the covalent attachment of single or multiple ubiquitin molecules to a protein, generating mono- and poly-ubiquitination respectively. Ubiquitin is a highly conserved protein of 76 amino acids (8 kDa) expressed in almost all eukaryotic cells. Ubiquitination takes place through three sequential steps: (i) an ubiquitin-activating enzyme (E1) catalyzes the activation of an ubiquitin molecule in its C-terminal glycine leading to a high-energy thiol ester intermediate; (ii) an ubiquitin-conjugating enzyme (E2) catalyzes the transfer of the activated ubiquitin to a member of the ubiquitin-protein ligase (E3) family; (iii) E3 promotes the transfer of the ubiquitin to a lysine residue in the target protein. Poly-ubiquitination requires the transfer of additional ubiquitin moieties to Lys48 of the previously conjugated ubiquitin molecule [96]. While mono-ubiquitination is involved in modulating cellular events, poly-ubiquitination targets the substrate proteins to the 26S proteasome for degradation [97]. Defects in the ubiquitination pathway have been associated with the etiology of a number of neurodegenerative disorders. Many neurological diseases are indeed characterized by the accumulation of aggregated misfolded proteins and inclusion bodies. These aggregates contain ubiquitin and proteasome subunits implying a failure of the UPS in the removal of misfolded proteins.

HD is characterized by the presence of intracellular aggregates of HTT protein with an expanded polyglutamine repeat [98,99]. HTT is ubiquitinated at Lys48 and Lys63 but the proteasome might not be able to digest extended polyQ sequences [100,101]. Moreover, HTT mutants were shown to sequester components of the proteasome within aggregates, possibly enhancing UPS inhibition [102]. Consistently with this model, proteasome activation ameliorates HD phenotype both *in vitro* and *in vivo* [103,104].

Similar to HD, ubiquitin was detected in NFTs and senile plaques from AD patients [105]. Remarkably, Aβ was demonstrated to inhibit the proteolytic activity of 26S proteasome, leading to a dramatic increase of ubiquitinated proteins in neurons [106,107]. Such effect seems due to the completion of Aβ oligomers with the physiological proteasomal substrates [108]. Also mutations in the *ubiquitin B* gene itself may contribute to AD phenotype. The frameshift mutant ubiquitin-B+1 (UBB+1) was indeed detected in AD brain and UBB(+1)-capped unanchored polyubiquitin chains were proposed to inhibit the 26S proteasome via a dominant negative mechanism of action [109,110,111].

### *6.1. SUMOylation*

Similar to ubiquitination, SUMOylation involves the covalent attachment of a member of SUMO (small ubiquitin-like modifier) family to lysine residues in specific target proteins. This PTM depends on an enzymatic cascade analogous to, but distinct from, the ubiquitination pathway [112]. Unlike ubiquitination, SUMOylation does not target proteins for degradation but regulates a wide variety of functions including protein stability, nuclear-cytosolic shuttling and transcription. SUMOylated proteins have been detected in neuronal inclusions associated with different misfolding diseases, suggesting a role in neurodegeneration.

As shown in polyQ diseases, SUMOylation affects neurotoxicity through different pathways. Among others, SUMO-mediated changes in protein transport across the nuclear membrane seem to be a contributory factor for neurodegeneration. The best example for this scenario is SCA1. In facts, ATXN1 can be SUMOylated on at least 5 different Lys residues (Lys16, Lys194, Lys610, Lys697 and Lys746) and the process is dependent on a functional nuclear localization signal and phosphorylation at Ser776. The polyQ expansion of ATXN1 was shown to decrease SUMOylation levels, possibly affecting its correct trafficking between the cytosolic and nuclear compartment upon disease [113]. SUMOylation can also alters the transcriptional process, as shown by the ability of a SUMOylated pathogenic fragment of HTT in repressing transcription and exacerbating neurodegeneration in a Drosophila model for HD [114]. Additionally, SUMOylation negatively regulates the intrinsic transcriptional activity of androgen receptor [115]. By disrupting SUMOylation patterns, transcriptional function of mutant androgen receptor-mediated disease ameliorates [116]. Lastly, SUMOylation can also affect aggregate formation as proven in DRPLA. The overexpression of SUMO1 in a neuronal model of the disease was shown to accelerate mutant ATN1 intranuclear aggregation and apoptosis [117].

## 7. Deamidation

Deamidation is the only PTM that does not require enzymatic catalysis. This spontaneous and irreversible reaction involves the cleavage of ammonia from the amide group of asparagine residues and to a lesser extent of glutamines [118]. In physiological conditions, deamidation of asparagines proceeds through formation of a five membered succinimide ring intermediate by the nucleophilic attack of the nitrogen atom in the following peptide bond on the carbonyl group of the asparagine side chain. The intermediate then undergoes hydrolysis to generate a mixture of aspartate and isoaspartate in a ratio of 1:3. *In vivo*, isoaspartate can be converted back to normal aspartate by the enzyme protein L-isoaspartyl O-methyltransferase (PIMT) [119]. Deamidation was initially considered as a mere form of molecular damage associated with aging [120]. However, a more complex role in regulating protein function and stability has been recently proposed. Moreover, an imbalance in the physiological aspartate:isoaspartate ratio—possibly due to PIMT deficits—has been associated with several pathological conditions including AD, vascular dementia (VaD) and multiple sclerosis (MS).

In AD context, mass spectrometry analysis of aggregated tau species from NFTs revealed the presence of isoaspartates at the level of Asp193, Asn381 and Asp387 as a consequence of deamidation and/or isomerization [121]. Interestingly, these isoaspartates are located in close proximity of known pathological hyper-phosphorylation sites, suggesting that isoaspartate

conversion might induce structural rearrangements that could in turn facilitate the phosphorylation process. Recently, extensive deamidation has been shown to take place also at Asn279 within the microtubule-binding repeat domain. Notably, this modification abrogates tau immunoreactivity to RD4 antibody—which recognizes the 4R isoform—and affects tau ability to bind microtubules [122]. Altogether, these findings highlight the importance of deamidation for tau self-assembly into PHFs. Furthermore, the repair enzyme PIMT was shown to co-localize with NFTs [123], corroborating its role in counteracting the accumulation of isoaspartate residues in aged proteins. Consistently, PIMT-deficient mice show aberrant increase of isoaspartates in the brain already at neonatal stages and die within 12 weeks of age due to fatal epileptic seizures [124].

VaD is a form of dementia caused by an insufficient supply of blood to the brain due to a series of small strokes [125]. Quantitative mass spectrometry profiling on VaD brain samples highlighted significant up-regulation of Na+/K+-ATPase ion channel and PIMT compared to controls. Moreover, increased deamidation was identified in the ion channel catalytic subunits ATP1A1 and ATP1A2. In particular, three deamidated residues (Asn210, Gln220 and Asn715) were found located in close proximity to the catalytic site of the channel, possibly impairing its correct functioning [126]. As ion channel proteins play a key role in maintaining the electrical cellular potential, aberrant deamidation may contribute to VaD pathogenesis via perturbation of membrane excitability and neuronal activity.

In the context of autoimmunity, aberrant protein deamidation may disturb the delicate balance that the immune system establishes between tolerance to self-proteins and the ability to respond to foreign antigens. Indeed, asparagine deamidation has been shown to interfere with antigen processing by the enzyme asparagine endopeptidase (AEP), contributing to diminished antigen presentation [127]. With regards of MS, deamidation of Asn94 in myelin basic protein (MBP) —a strong candidate auto-antigen—has been hypothesized to allow the survival of the $MBP_{85\text{-}99}$ peptide, which might trigger the pathological immune response [127,128]. Additionally, differential deamidation between MS cases and controls was observed at Gln8, Gln103 and Gln109 [129].

## 8. Oxidative/Nitrosative Stress and PTMs

Oxidative stress can modify numerous biological molecules via redox-mediated reactions [130]. Among others, oxidative stress is able to induce a number of PTMs through direct oxidation of protein side-chains by reactive oxygen and nitrogen species [131]. The main radical species involved in such modifications are superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), the highly reactive hydroxyl radical ($HO^\bullet$), and nitrogen species such as nitric oxide (NO). These PTMs can be either irreversible or reversible. The former include carbonylation and tyrosine nitration, while the latter include cysteine modification products such as S-sulfenation, S-nitrosylation, S-glutathionylation and disulfides [132]. Oxidative stress is the result of an imbalance between biochemical processes leading to production of free radical species and those responsible for their removal, the cellular antioxidant cascade. For instance, dyshomeostasis of metal ions, such as copper, manganese, iron and other trace redox-active transition metals, affects metalloproteins activity and in turn increase free radical production [133,134]. Although radical species can exert regulatory functions [135,136], their production is usually associated with aging and numerous pathological states including neurodegenerative disorders.

**Table 1.** Principal S-nitrosylated proteins in Alzheimer's and Parkinson's diseases.

| Protein | Biological function | Effect of S-nitrosylation |
|---|---|---|
| Parkin | E3 ubiquitin ligase participating in the ubiquitin-proteasome system [156] | Altered E3 ligase activity and protein misfolding [149] |
| Protein disulfide isomerase (PDI) | Molecular chaperone [157] | Inhibition of its chaperone activity and protein misfolding [149,150] |
| Dynamin-related protein-1 (DPR1) | GTPase involved in normal mitochondrial fission [158] | GTPase overactivation and excessive mitochondrial fission [149,150] |
| Serine/threonine kinase CDK5 | Brain development, neuronal differentiation and migration, axon guidance, synaptic plasticity [159] | Upregulation of its kinase activity and increased dendritic spine loss and neuronal apoptosis [150] |
| Peroxiredoxin-2 (PRX2) | Antioxidant protein reducing intracellular peroxides [160] | Inhibition of its antioxidant activity [149] |
| Matrix metalloproteinase-9 (MMP-9) | Breakdown of extracellular matrix [161] | Metalloproteinase over-activation, cell damage and death [149] |
| Cyclooxygenase-2 (COX-2) | Prostaglandin-endoperoxide synthase responsible for prostaglandin (PG)H2 formation [162] | Activation of its prostaglandin synthesis activity, contribution to the neuroinflammatory component of neurodegeneration [149] |
| Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | Glycolytic enzyme [163] | Enhanced Siah1 binding, p300/CBP activation and increased neuronal death [149,150] |
| Parkinson disease protein 7 (DJ-1) | Protein deglycase that repairs methylglyoxal- and glyoxal-glycated amino acids and proteins [164] | Possible alteration of its anti-cell death activity [149] |
| Microtubule-associated protein 1B (MAP1B) | Microtubule-binding protein which actively extends axon length [165] | Increased microtubule binding and its own degradation; increased axonal retraction; decreased neuronal death [150] |
| X-linked inhibitor of apoptosis (XIAP) | Protein with antiapoptotic properties [166] | Inhibition of E3 ligase and anti-apoptotic activity; increased caspase activity, increased neuronal death [149,150] |
| Phosphatase and tensin homolog (PTEN) | Dephosporylation of 3' phosphate of the inositol ring in PIP3, resulting in inhibition of the AKT signaling pathway [167] | Inhibition of its phosphatase activity [149] |
| NMDA Receptor (NMDAR) | Synaptic plasticity and memory functions [168] | Excitotoxicity and neuronal cell death [150] |

Protein carbonylation and tyrosine nitration are often associated with oxidative damage and used as biomarkers for assessment of oxidative stress in aging and diseases [137]. Conversely from

the other PTMs, carbonylation can occur on several amino acids residues, including arginines, histidines, lysines, prolines, threonines and cysteines. Carbonylated proteins have been detected in several neurodegenerative disorders including AD, PD, MS and amyotrophic lateral sclerosis (ALS). Cytoskeletal components such as neurofilaments and tubulin seem to be the major targets for carbonylation [138]. Interestingly, besides its detrimental effects, protein carbonylation plays also a role in signal transduction [139,140] and exerts protection against ischemia-reperfusion injury [141]. Tyrosine nitration, usually 3-nitrotyrosine, is a highly selective process since only well-defined tyrosine residues can be nitrated. This PTM is associated with different neurodegenerative disorders as well as acute or chronic inflammation [142]. In AD, tau nitration at Tyr29 has been associated with NFT formation while nitration at Tyr18 and Tyr394 was shown to reduce tau self-assembly *in vitro* [143,144]. In PD, α-synuclein nitration seems to promote LB formation via decreasing its solubility [145,146,147].

S-nitrosylation is a redox-mediated PTM that modulates protein function by covalent addition of NO to thiol groups of cysteine residues. In physiological conditions, S-nitrosylation formation is counterbalanced by denitrosylation enzymes—such as S-nitrosoglutathione reductase, protein disulphide isomerase and the thioredoxin system—and by protein-protein transnitrosylation [148,149]. Aberrantly S-nitrosylated proteins with synaptic function and neuronal survival roles have been found in AD [150] and PD [151] (**Table 1**). Altered S-nitrolyation levels in these proteins may impact a variety of cellular mechanisms encompassing synaptic transmission, mitochondrial function, iron homeostasis, receptors and ion channels, protein quality control and transcription factors [149]. In addition, nitrosative and oxidative stress seem to contribute to protein misfolding and aggregation via chaperone and proteasomal dysfunctions (**Table 1**).

Cysteines are molecular switches highly reactive to environmental redox conditions. They can be found in either free form or disulfide bond. Changes in their state cause structural and functional modifications within proteins. To date, the role of disulfide bond formation in neurotoxicity is still controversial. For instance, it has been reported that the reduced form of $PrP^C$ causes the loss of secondary structure and disrupts native tertiary interactions [152], suggesting a role for disulfide bond in preventing $PrP^C$ misfolding [153]. In contrast, we have recently shown that reducing the disulfide bond of $PrP^C$ globular domain increases β-sheet content and hydrophobic surfaces exposed to the solvent [154,155]. Differences in the experimental setups might account for these opposite outcomes.

Taking together, these pieces of information highlight the multiple effects of oxidative stress on neuronal physiology. In particular, oxidative stress can affect cell functioning and viability by directly altering protein stability and folding or modifying protein activity.

## 9. Conclusion

Despite great advances made in recent years to understand the role of PTMs in neuronal physiology and pathology (**Figure 1**), we are still far from picturing the global PTM framework in terms of complexity and regulation. In the next future, two goals should be pursued: (i) characterization of all PTMs and the interplay between them; (ii) identification of combinatorial patterns of modification in different physiological conditions—the so called "PTM code"—for providing information on protein state [169]. For these purposes, technological advances in both experimental setup and *in silico* prediction tools are required. Concerning the experimental setup, it

should be taken into consideration that most PTMs are reversible and thus susceptible to the methodological approach used. Most of them are indeed removed or altered during sample preparation, so generating artifacts or contradictory results. New protocols taking into account reversibility of modifications as well as new methodological approaches for *in situ* evaluation of PTMs should be developed.

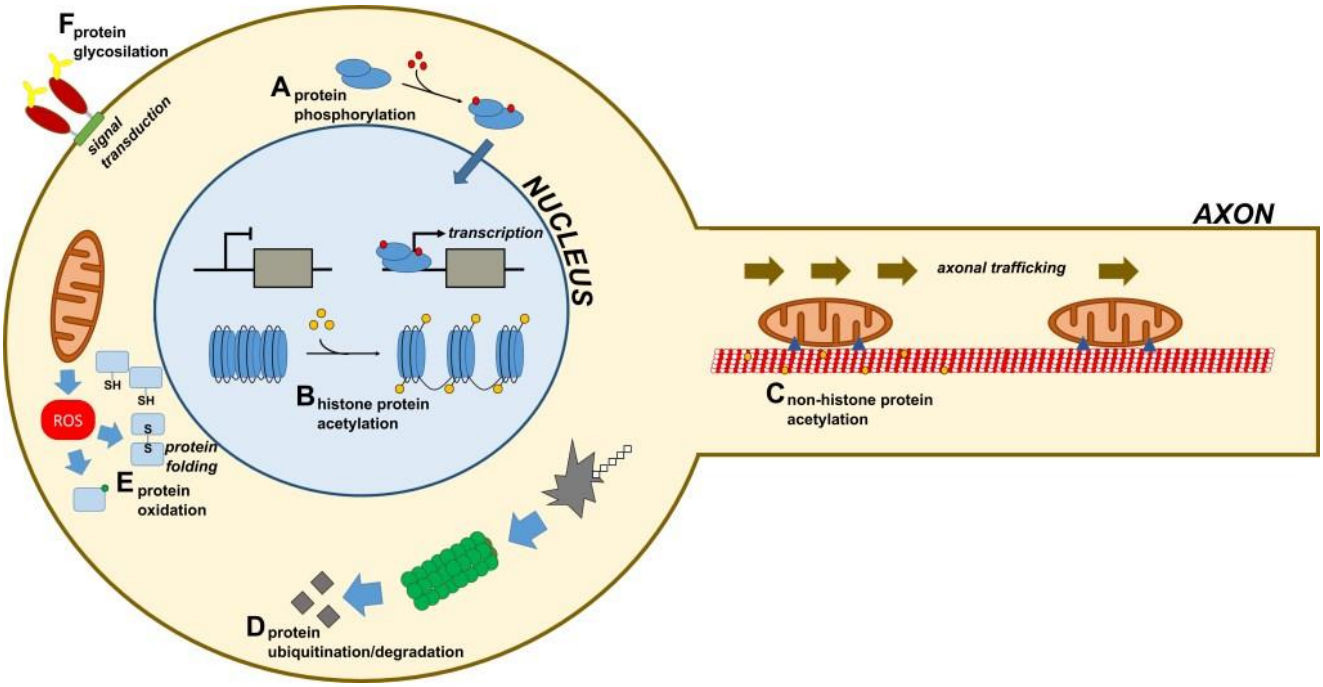


**Figure 1.** The role of post translational modifications (PTMs) in neuronal physiology and pathology. PTMs regulate several cellular processes in neurons. Protein phosphorylation (A) modulates the conformation and localization of target proteins, affecting a myriad of functions such as transcription. Acetylation of histone proteins (B) controls chromatin conformation and in turn gene expression. Acetylation of non-histone proteins such as tubulin (C) modulates microtubule-dependent axonal transport and eventually synaptic transmission. Protein ubiquitination (D) promotes protein degradation through the proteasome; it is important to remove defective or incorrectly folded proteins. The activity of reactive-oxygen species (ROS) (E) can affect the correct function of proteins by inducing their dimerization through the formation of disulfide bonds or their carbonylation/nitrosylation. Glycosylation (F) can modulate intracellular signaling by controlling the subcellular localization of target proteins and their sets of biological interactions.

Upon disease, PTMs are perturbed in response to upstream pathological events affecting the concerted action of opposite classes of enzymes. Identification of molecular mechanisms leading to an aberrant regulation of PTMs in the nervous system and disclosure of PTM code will be crucial for understanding the etiology of the different neurodegenerative disorders as well as for developing therapeutic strategies. In facts, unveiling the cellular pathways and the molecular players that are affected upon disease might provide novel druggable targets for therapies aimed at restoring the physiological PTM code in neurons.

## Conflict of Interest

All authors declare no conflicts of interest in this paper.

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