The Ubiquitin System in Alzheimer's Disease

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Abbreviations

Aβ: Amyloid beta
AD: Alzheimer's disease
DLB: Dementia with Lewy Bodies
DUB: Deubiquitinase
E1: E1-activating enzyme
E2: E2 conjugating enzyme
E3: E3 ubiquitin ligase
E-NFTs: Extracellular neurofibrillary tangles
FTD: Frontotemporal dementia
FTD-U: Frontotemporal dementia with ubiquitin pathology
HECT: Homologous to E6AP carboxyl terminus
HD: Huntington's Disease
IGF: Insulin-like growth factor
LTP: Long term potentiation
MAM: Mitochondria-associated membranes
MLKL: Mixed lineage kinase domain-like
mTOR: Mammalian target of rapamycin
NEDD4: neuronal precursor cell-expressed developmentally down-regulated 4
NFTs: Neurofibrillary tangles
PHFs: Paired-helical filaments
PM: Plasma membrane
PROTACs: proteolysis targeting chimeric molecules
PSD: Postsynaptic densities
PTM: Post-translational modification
RING: Really-Interesting new gene
RIPK: Receptor-interacting serine/threonine protein kinase
RBR: Ring-between-ring
SCF: Skp, Cullin, F-box containing E3 ligase complex
USP: Ubiquitin-specific protease

Abstract

Alzheimer's Disease (AD) is the most common form of dementia, most prevalent in the elderly population and has a significant impact on individuals and their family as well as the health care system and the economy. While the number of patients affected by various forms of dementia including AD is on the increase, there is currently no cure. Although genome-wide association studies have identified genetic markers for familial AD, the molecular mechanisms underlying the initiation and development of both familial and sporadic AD remain poorly understood. Most neurodegenerative diseases and in particular those associated with dementia have been defined as proteinopathies due to the presence of intra and/or extracellular protein aggregates in the brain of affected individuals. Although loss of proteostasis in AD has been known for decades, it is only in recent years that we have come to appreciate the role of ubiquitin-dependent mechanisms in brain homeostasis and in brain diseases. Ubiquitin is a highly versatile post-translational modification which regulates many aspects of protein fate and function, including protein degradation by the Ubiquitin-Proteasome System (UPS), autophagy-mediated removal of damaged organelles and proteins, lysosomal turnover of membrane proteins and of extracellular molecules brought inside the cell through endocytosis. Amyloid-β (Aβ) fragments as well as hyperphosphorylation of Tau are hallmarks of AD, and these are found in extracellular plaques and intracellular fibrils in the brain of individuals with AD, respectively. Yet, whether it is the oligomeric or the soluble species of Aβ and Tau that mediate toxicity is still unclear. These proteins impact on mitochondrial energy metabolism, inflammation, as well as a number of house-keeping processes including protein degradation through the UPS and autophagy. In this chapter, we will discuss the role of ubiquitin in neuronal homeostasis as well as in AD; summarise crosstalks between the enzymes that regulate protein ubiquitination and the toxic proteins Tau and Aβ; highlight emerging molecular mechanisms in AD as well as future strategies which aim to exploit the ubiquitin system as a source for next generation therapeutics.

1. Alzheimer's Disease

1.1. Dementia

Neurodegeneration is an umbrella term used to describe the degeneration and eventual death of neurons which usually occurs late in life or in individuals with an associated neurodevelopmental defect. Amongst neurological disorders, dementia is caused by the progressive loss of cognitive functions which leads to symptoms such as memory loss and disorientation. The main differentiating factor that assists in characterising the various forms of dementia is whether the pathology primarily affects the cortical regions (outer layer of the cerebrum) or non-cortical regions (involving the thalamus, basal ganglia and vasculature). Cortical dementias include Alzheimer's Disease (AD) which is the most common form of dementia with around two-thirds of cases, Vascular Dementia (VD) which accounts for 20% of dementia cases, dementia with Lewy bodies (DLB) which represents 10-15% of cases, and Frontotemporal Dementia (FTD) with less than 5% of dementia cases (Alzheimer's-Association, 2014). AD is defined by a progressive decline in mental, behavioural and cognitive functions, with a median survival time from diagnosis of less than five years (Xie *et al*, 2008). FTD, commonly termed Pick's Disease, can be easily confused with AD at the time of diagnosis. Both dementias are characterised by progressive cognitive decline, the build up of aggregated proteins and cortical atrophy. However, FTD can occur in younger individuals (above 35 years age) and is detected by more rapid, extreme changes in personality. FTD-U is distinct from FTD in the fact that that it contains inclusions which stain positive for the small protein modifier ubiquitin but not for Tau, α-synuclein or polyglutamine antibodies (Rosso *et al*, 2001; Kertesz *et al*, 2000). In contrast, Parkinson's Disease (PD), Huntington's Disease (HD) and progressive supranuclear palsy disorders affect subcortical structures. Both DLB and PD are characterised by α-synuclein inclusions although other studies have suggested that these aggregates can also be found in other neurodegenerative diseases including familial and sporadic AD (Spillantini *et al*, 1998; Holmes *et al*, 1999; Spillantini & Goedert, 2000). PD dementia (PDD) is characterised by a slow progressing dementia with impairment in memory and executive functions, and it is usually observed after 10 years of diagnosis (Emre *et al*, 2007). Individuals exhibiting signs of cognitive decline within 1-year of PD diagnosis are likely exhibiting DLB rather than PDD.

1.2. Genome-Wide Association Studies (GWAS)

Genome-Wide Association Studies (GWAS) have identified genetic variants, or single nucleotide polymorphism (SNPs), which may be associated with familial AD mostly. The original genetic studies identified mutations in amyloid precursor protein (*APP*) (Goate *et al*, 1991) and Presenilin (*PSEN1/PSEN2*) (Sherrington *et al*, 1995) as genetic risk factors for familial early-onset AD. In contrast, genetic loci for late-onset AD include *TREM2* (Jonsson *et al*, 2013; Guerreiro *et al*, 2013; Carmona *et al*, 2018), *ABCA7* (Steinberg *et al*, 2015), *TP53INP1* and *IGHV1* (Escott-Price *et al*, 2014), phospholipase D3 (Cruchaga *et al*, 2014). Recent comprehensive meta-analysis identified 29 risk loci and 215 potential causative genes, suggesting roles for the immune system, lipid-related

processes, APP and protein degradation in AD (Jansen *et al*, 2019; Kunkle *et al*, 2019). GWAS have also identified specific genetic variants in different types of dementia including in FTD (Ferrari *et al*, 2014) and DLB (Guerreiro *et al*, 2018), and have also revealed potential overlap between the genetic make up of dementia and Parkinson's Disease (Guerreiro *et al*, 2016; Ferrari *et al*, 2017).

GWAS have been less consistent for sporadic AD, for which aging remains the greatest risk factor. Nevertheless, Apolipoprotein Epsilon 4 (*APOE ε4*) has emerged as the most prevalent genetic risk factor for both familial and sporadic late-onset AD (Poirier *et al*, 1993; Raber *et al*, 2004). ApoE regulates lipid homeostasis by mediating lipid, fat-soluble vitamin and cholesterol transport between cells and tissues. In the brain, it is produced and secreted by astrocytes and recognised by the ApoE receptor on the surface of neurons (Lane-Donovan & Herz, 2017). The *APOE ε4* variant is less efficient than other alleles during neuronal repair, and this may increase susceptibility to AD.

Current estimates suggest that by 2050 the number of AD cases will have reached 115 million worldwide, which represent a three-fold increase compared to 2010 (Prince *et al*, 2013). Yet, the development of AD drugs showed a 99.6% failure rate between 2002 and 2012 (Cummings, 2018). The impending dementia epidemic is being closely monitored at the global level, and research priorities have now been identified, beyond the amyloid hypothesis. Amongst these, fundamental research into the basic mechanisms of dementia has been highlighted as holding the greatest potential for identifying novel drug targets for AD (Prince *et al*, 2016; Shah *et al*, 2016).

1.3. Molecular mechanisms in AD

Amyloid-beta (Aβ)

The amyloid precursor protein (APP) is a transmembrane glycoprotein found in human, *C. elegans* (APL-1) and *D. melanogaster* (APPL) but not in prokaryotes, plants or yeast (Kang *et al*, 1987). A current hypothesis suggests that *APP* has been acquired during evolution at the same time as the development of a central nervous system with functioning synapses (van der Kant & Goldstein, 2015). The human *APP* gene contains 18 exons and is prone to splicing. In fact, tissue-specific *APP* variants have been identified in smooth, cardiac and skeletal muscle, as well as organs such as the kidney and pancreas. The brain-specific *APP* variant excludes exon 7 and 8, which suggests that the function of APP proteins is tissue specific. Of the three major splice isoforms of *APP* (APP_{695}, APP_{751}, APP_{770}), APP_{695} is the predominant neuronal form. Studies in the invertebrate models *C. elegans* and *D. melanogaster* have revealed that *APP* orthologues play a role in axonal transport and neuronal signaling. The conditional knockout of *APP* in mouse brains results in long term potentiation (LTP) phenotypes strongly suggesting that APP plays a role in maintaining neuronal plasticity and brain homeostasis (Seabrook *et al*, 1999).

APP proteolytic processing is key to its function in regulating neuronal activity (Kamenetz *et al*, 2003), and can occur through the amyloidogenic and non-amyloidogenic pathways (Sisodia, 1992; De Strooper *et al*, 1998; Vassar *et al*, 1999). The non-amyloidogenic pathway results in the initial

cleavage of APP via α-secretase (Esch *et al*, 1990), resulting in a truncated APP protein that lacks the Aβ N-terminus. Subsequent cleavage via γ-secretase results in the non-pathogenic truncated Aβ peptide p3 and the amyloid precursor protein intracellular domain (AICD) (Haass *et al*, 1993). In the amyloidogenic pathway, APP is cleaved by the β-secretase enzyme BACE which releases sAPPβ. It is the second cleavage by γ-secretase which leads to the intracellular release of AICD in the nucleus and of extracellular Aβ peptides (40 and 42). Aβ peptides can aggregate into oligomers to form fibrils and ultimately plaques (Hardy & Higgins, 1992). Interestingly, the release of Aβ peptides into the brain interstitial fluid is activity-dependent and requires APP endocytosis and its processing by the endocytic recycling machinery, which emphasizes the role of APP processing and Aβ in normal neuronal activity (Cirrito *et al*, 2008; Choy *et al*, 2012). In contrast to Aβ peptides, sAPPα has been shown to bind BACE and decrease soluble Aβ and Aβ plaques (Obregon *et al*, 2012). Since plaques are also found in the brain of elderly AD-free individuals, the toxic potential of these protein aggregates remains unclear. Indeed, soluble oligomeric pools of Aβ have also been implicated in driving the neurotoxic effects of Aβ (McLean *et al*, 1999). Interestingly, recent studies have shed light on the dynamic nature of Aβ conformations, by showing that plaques can be readily removed from the brain during sleep, and that sleep disturbance may affect metabolite clearance and can lead to Aβ accumulation (Xie *et al*, 2013; Shokri-Kojori *et al*, 2018).

Tau

In contrast to APP which is an integral membrane protein, Tau is a cytosolic protein found to bind to and stabilise mitocrotubules (Goedert *et al*, 1988). Human Tau consists of six isoforms with varying amino acid chain lengths. These isoforms have a microtubule binding domain which consist of C-terminal repeats (R1-R4) and an N-terminal projection domain composed of N1 and N2 motifs. The projection domain mediates interaction with the neural plasma membrane as well as cytoskeletal proteins through the microtubule binding protein MAP1A (Himmler *et al*, 1989; Buée *et al*, 2000). Tau isoforms are named according to the presence of these motifs and include 2N4R, 1N4R, 0N4R, 2N3R, 1N3R and 0N3R (Cowan & Mudher, 2013). The balance between 3R and 4R isoforms is lost in tauopathies and Tau mouse models and this leads to Tau hyperphosphorylation, insolubility and in turn cognitive impairment (Espíndola *et al*, 2018). In the Tau hypothesis, hyperphosphorylated Tau accumulates into pathological inclusions and tangles which are observed in AD (Goedert & Jakes, 1990). Protein phosphorylation leads to Tau dissociation from microtubules and the formation of oligomeric species that further aggregate into paired-helical filaments (PHFs) and neurofibrillary tangles (NFTs) (Chirita *et al*, 2005; Patterson *et al*, 2011). Although the pathological trigger leading to hyperphosphorylated Tau is still poorly understood, the main kinases responsible for its phosphorylation have been identified and include Cyclin Dependent Kinase 5 (CDK5), Mitogen Activated Protein Kinase (MAPK) (Baumann *et al*, 1993) and Glycogen synthase kinase 3 (GSK3) (Hooper *et al*, 2008). Hyperphosphorylated Tau, which exists in a soluble oligomeric form, mediates neuronal toxicity by breaking down microtubules and impairing axonal transport (Cuchillo-Ibanez *et al*, 2008; Cowan *et al*, 2010; Yamada *et al*, 2015).

Loss of proteostasis in AD

Seminal studies in *C. elegans* and *D. melanogaster* have established that Insulin Growth Factor (IGF) and mTOR pathways regulate lifespan (Kenyon, 2010). In *C. elegans* for example, *daf-2* (IGFR in human) mutants are long-lived and stress-resistant. Given that aging is the most reliable risk factor for sporadic AD, it is perhaps not surprising that these pathways, through their control of protein synthesis, play important roles in AD. In support of this, *daf-2* RNAi reduces Aβ42-induced proteotoxicity (Cohen *et al*, 2006). Protein synthesis is inherently error-prone and an estimated 30-90% of newly synthesized proteins are defective and improperly folded (Rothman, 2010). In eukaryotic cells, the Ubiquitin-Proteasome System (UPS), the endosomal-lysosomal pathway and autophagy participate in protein quality control to prevent the accumulation of non-functional and misfolded proteins (Taylor & Dillin, 2011). The endosomal-lysosomal pathway for instance handles monomeric Aβ42, while autophagy and the UPS clear Aβ42 oligomeric species (Ji *et al*, 2018).

The first link between proteasomal dysfunction and AD came from histopathological analysis of protein aggregates from AD brains, which revealed the presence of the small protein modifier ubiquitin (Perry *et al*, 1987; Mori *et al*, 1987; Kuzuhara *et al*, 1988). GWAS studies have also added to mounting evidence in support for a role of altered ubiquitin signaling, with the discovery of E3 ubiquitin ligases TRIM15 (Shi *et al*, 2010) and UBR5 as potential genetic markers (Hu *et al*, 2011). In addition to this, ubiquitin (section 3.3), as well as a growing number of E3 ligases and deubiquitinases, have also been implicated in AD (**Table 1**). Proteomics has also been particularly useful in order to identify changes that occur at the proteome level during AD progression. This include for example the remodeling of UCHL5 interactome (Kikuchi *et al*, 2013), alterations in overall protein ubiquitination (Tramutola *et al*, 2018) and overall protein levels (Manavalan *et al*, 2013).

2. The Ubiquitin Proteasome system

2.1. Ubiquitin

Ubiquitin is a highly conserved 76kDa protein modifier found in all eukaryotes. The yeast *Saccharomyces cerevisiae* ubiquitin sequence varies by only 2 amino acids compared to the mammalian one (Varshavsky, 2001). In contrast, ubiquitin is not found in prokaryotes and instead prokaryotic ubiquitin-like protein (Pup) and Small Archaeal Modifying Protein (SAMP) drive protein degradation (Pearce *et al*, 2008; Humbard *et al*, 2010). Ubiquitin is a versatile post-translational modification (PTM) which mediates a plethora of functions including protein degradation as part of the UPS but also proteasome-independent functions (Hershko & Ciechanover, 1998; Komander, 2009). These include the sorting of receptors at endosomes (Clague *et al*, 2012), the recognition of protein complexes and organelles by autophagic receptors (Deng *et al*, 2017), as well as the recruitment of the DNA repair machinery at sites of DNA double strand brakes (Schwertman *et al*, 2016). In addition to ubiquitin, mammalian cells also encode a number of Ubiquitin-Like modifiers (UBLs) such as SUMO (Flotho & Melchior, 2013), NEDD8 (Enchev *et al*, 2015), and ISG15 (Hermann & Bogunovic, 2017).

The structure of ubiquitin is characterized by a β-grasp fold and a flexible six-residue C-terminus tail which is important for its conjugation onto lysines of protein substrates (Vijaykumar *et al*, 1987). The hydrophobic patch formed by Ile 44, Leu 8, Val 70 and His 68 is key for its recognition by most ubiquitin binding domains (UBDs) (Sloper-Mould *et al*, 2001). The hydrophobic surface which centers around Ile 36 (Ile 36, Leu 71 and Leu 73), seems to be particularly important for the transfer of ubiquitin between E2-conjugating enzymes and E3 ubiquitin ligases (Kamadurai *et al*, 2009) as well as for its interaction with UBDs (Reyes-Turcu *et al*, 2006) and DUBs (Deubiquitinating enzymes) (Hu *et al*, 2002; Hospenthal *et al*, 2013). Protein ubiquitination can result in the addition of one molecule of ubiquitin onto a single lysine (i.e. monoubiquitin) or on multiple lysines residues (multi-monoubiquitination). In addition to this, multiple ubiquitin molecules can be assembled together to form a ubiquitin chain. This is mediated between the C-terminal glycine residue (G76) on a donor ubiquitin and the free amino group of the N-terminal methionine (M1) or of any of the seven lysines from an acceptor ubiquitin. Therefore, as many as eight distinct linkages can be formed between two ubiquitin molecules and these include Met-1, K6, K11, K27, K29, K33, K48 and K63 (Hershko & Heller, 1985; Komander & Rape, 2012). Although the canonical ubiquitin signal that mediates protein degradation via the UPS was originally found to be K48-linked polyubiquitin chains, additional ubiquitin chain types including the more complex mixed and branched chains have since now been reported (Chau *et al*, 1989; Yau & Rape, 2016). This, together with the fact that ubiquitin itself can be modified with other PTM, further expands the complexity and functional diversity of ubiquitin.

2.2. Components of the ubiquitin system

Ubiquitin is covalently tagged onto target proteins via an enzymatic cascade which include E1-activating, E2-conjugating and E3 ubiquitin ligases. The C-terminal glycine residue of ubiquitin is first adenylated in the presence of ATP, followed by its transfer onto the catalytic cysteine (Cys) residue of an E1-activating enzyme via a thioester bond (Ciehanover *et al*, 1978; Hershko *et al*, 1981). The activated ubiquitin is then transferred in a transthioesterification reaction onto the catalytic residue of an E2-conjugating enzyme, prior to its loading onto lysines residues of protein substrates (Hershko *et al*, 1980). E3 ubiquitin ligases mediate this final step, enabling the formation of an isopeptide bond between the carboxyl group of G76 of ubiquitin and the free amino group on lysine(s) of a protein substrate (Hershko *et al*, 1979).

Depending on the mechanism of ubiquitin transfer onto the substrate, E3 ligases have been divided into three main families. Really interesting new gene (RING), characterised by their RING finger zinc domain or U-box domain, are E3 ligases that act as scaffolds to facilitate the transfer of ubiquitin from the E2, directly to the target substrate (Lorick *et al*, 1999). RING finger domains have been shown to adopt a “cross-brace” arrangement through binding two zinc ions (Zheng *et al*, 2000). RING fingers have also been suggested to activate E2s allosterically (Ozkan *et al*, 2005). There are over 600 RING E3s encoded in the mammalian genome, making this family of E3s the most diverse (Li *et al*, 2008). The fact that BRCA1/BARD1 assembles different ubiquitin chains depending on the E2

it partners with, has led to the recognition that, in the case of RING E3s, polyubiquitin chain specificity is primarily determined by E2s (Christensen *et al*, 2007). In contrast, Homologous to E6AP carboxyl terminus (HECT) E3s show intrinsic catalytic activity by accepting ubiquitin from the E2, before it is transferred to the substrate (Huibregtse *et al*, 1995). The C-terminal HECT domain is characterised by an N- and a C-lobe, connected by a flexible glycine hinge region linking the lobes, and with the catalytic cysteine residue located on the C-lobe (Huang *et al*, 1999). In contrast to RINGs E3s, polyubiquitin chain specificity in HECT ligases is determined by the HECT domain itself, as changing the E2 that interacts with the HECT domain does not affect ubiquitin linkage type (Kim & Huibregtse, 2009). RING-in-between-RING (RBR) E3 ligases represent a hybrid family between RINGs and HECTs. They have a RING domain for E2 recruitment which subsequently transfers ubiquitin via transthioesterification onto a cysteine residue of a RING-like domain (Wenzel *et al*, 2011). PARK2 is perhaps the most relevant example for neurodegeneration, given that loss of Parkin ubiquitin ligase activity in PD and AD is associated with mitophagy inhibition and the accumulation of damaged mitochondria (Kitada *et al*, 1998; Narendra *et al*, 2008; Ye *et al*, 2015).

One of the main functions of ubiquitin is to label proteins for degradation by the 26S proteasome (Hershko & Ciechanover, 1992). The 26S proteasome is a multi-protein complex organized into the 19S regulatory cap and the 20S catalytic core which is responsible for the proteolytic cleavage of most intracellular and soluble proteins. The 20S core particle has two outer (α) and two inner (β) rings, with each ring composed of seven subunits. The catalytically active β-rings which make up the proteolytic region, consists of N-terminal nucleophile hydrolases, with threonine functioning as the active nucleophile (Lowe *et al*, 1995). These hydrolases include trypsin, chymotrypsin and peptidyl-glutamyl-peptide (Heinemeyer *et al*, 1997). The 19S regulatory lid on the other hand has multiple subunits serving as UBDs for the docking and processing of ubiquitinated substrates by the 20S. For example, Rpn10 is a 19S regulatory subunit which recognises polyubiquitin chains through its Ubiquitin-Interacting Motif (UIM) (Deveraux *et al*, 1994).

At least 99 DUBs are encoded in the human genome and these have been grouped into the JAMM/MPN, OTU, MJD, UCH, MINDY and ZUP1 subfamilies based on their catalytic domain, with new families still being discovered (Clague *et al*, 2019). Some DUBs, including those from the OTU family, have shown some remarkable specificity towards particular chain types (Licchesi *et al*, 2012; Clague *et al*, 2019) In addition to their role in editing and modulating cell signaling through reversal of protein ubiquitination, DUBs also have important roles at the surface of endosomes and on the 19S lid of the proteasome where they ensure the recycling of ubiquitin thereby maintaining the pool of ubiquitin in cells. Rpn11, Ubp6/USP14 and UCH37 are the resident proteasomal DUBs in charge with substrate deubiquitination prior to unfolding and degradation (Verma *et al*, 2002; Stone *et al*, 2004; Lee *et al*, 2011).

2.3. Ubiquitin signaling and neuronal homeostasis

Neuronal cells communicate through synapses, formed between the axon of a sending neuron and the dendrite of the receiving neuron, with the pre- and postsynaptic membranes separated by the synaptic cleft. Each of these compartments have a number of cell adhesion molecules and scaffold proteins which enable the release of neurotransmitter from the pre-synaptic density into the synaptic cleft, in response to an action potential (Südhof, 2018). Postsynaptic membranes contain the receptors that bind the released neurotransmitters, leading to the activation of downstream signaling pathways which regulate synaptic plasticity, learning and memory (Kelleher *et al*, 2004).

Glutamate is a major excitatory neurotransmitter in mammalian brains and the regulation of its receptors is crucial for neuronal plasticity. The binding of glutamate onto NMDA (N-methyl D-aspartate) receptors (NMDAR), triggers the opening of this ion conductance channel protein, and the influx of calcium. This increase in intracellular calcium levels is a key signaling event in neurotransmission and therefore it needs to be tighly regulated. Astrocytes play an important role in this process by metabolising excess extracellular glutamate at synapses (Südhof, 2012). In AD, it has been shown that Aβ can impede the function of neuronal receptors including NMDAR, AMPAR (α-amino-3-hyroxy-5-methyl-4-isoxazolepropionate acid receptor), the Prion Protein cell surface (PrP^C), as well as drive inflammation in astrocytes which further contributes to neurotoxicity (Sheng *et al*, 2012; Akama *et al*, 1998; Laurén *et al*, 2009; Freir *et al*, 2011). Loss of glutamate homeostasis at synapses leads to the activation of extrasynaptic NMDA receptors. The ensuing excitotoxicity is mediated by the stabilisation and activation of calpain-dependent cleavage of the striatal-enriched protein tyrosine phosphatase (STEP), the activation of p38 mitogen-activated protein kinases and neuronal apoptosis (Xu *et al*, 2009; Wang & Qin, 2010).

Calcium signaling is intimately linked with ubiquitin ligase function. For instance, the HECT E3 NEDD4 (neuronal precursor cell-expressed developmentally down-regulated 4) binds calcium through its N-terminal calcium-binding module (C2 domain). In the absence of calcium, the C2 domain binds to the C-terminal HECT domain of NEDD4, inhibiting its ubiquitin ligase activity. In contrast, the increase in intracellular calcium concentration outcompete this interaction and releases the HECT domain. NEDD4 then translocates to the plasma membrane where it ubiquitinates synaptic proteins and contributes to neuronal activity (Wang *et al*, 2010; Scudder *et al*, 2014).

Normal brain function requires a precise and dynamic control over the remodelling of synaptic signaling processes. Ubiquitin plays key roles in regulating synaptic receptors through proteasome-dependent and independent mechanisms (Ehlers, 2003; Ma *et al*, 2017). Both NMDA and AMPA receptors are regulated by protein ubiquitination (Colledge *et al*, 2003). For example, AMPA receptor surface expression is regulated by the opposing activities of the E3 ligase NEDD4 and the DUB USP8 (Ubiquitin-Specific Protease 8) (Lin *et al*, 2011; Hou *et al*, 2011; Dosemeci *et al*, 2013; Scudder *et al*, 2014). The NMDA receptor is a substrate of FBXO2 and MIB2 E3 ligases, and loss of ligase activity disrupts synaptic function (Jurd *et al*, 2008; Atkin *et al*, 2014). In addition to synaptic receptors, the UPS also regulates the turnover of postsynaptic scaffolding proteins such as PSD-95

(Colledge *et al*, 2003). Another interesting study showed that proteasomes are recruited to synapses, further implicating the UPS as a key regulator of synaptic proteins (Bingol *et al*, 2010). Together, these and other studies implicate ubiquitin signaling as an important regulator of the trafficking and turnover of ionotropic glutamate receptors during neuronal activity (Goo *et al*, 2015).

3. Ubiquitin signaling in Alzheimer's Disease

3.1. Ubiquitin and protein aggregation

Some of the earliest studies linking UPS dysfunction and AD pathology reported the accumulation of ubiquitin-bound proteins in NFTs (Mori *et al*, 1987; Perry *et al*, 1987). Ubiquitin was also found in neuritic plaques and NFTs in the cortex of AD brains, in Lewy bodies and filaments associated with PD and Pick's disease as well as HD (Cole & Timiras, 1987; Kuzuhara *et al*, 1988; Lowe *et al*, 1988; Mori *et al*, 1987). Furthermore, ubiquitin is detected in both intracellular inclusions as well as extracellular-NFTs (E-NFTs) in AD brains (Manetto *et al*, 1989; Tabaton *et al*, 1991). Components of the proteasome were also found in NFTs in AD and DLB (Fergusson *et al*, 1996). Whether these represent active or functional proteasomes particles is still unclear. Importantly, the role and function of protein ubiquitination with regards to aggregate-prone proteins is starting to emerge. NEDD4 was recently shown to ubiquitinate α-synuclein filaments, suggesting that other protein inclusions might also be targeted by ubiquitin-mediated clearance mechanisms (Mund *et al*, 2018). However, some types of protein aggregates might exert protective functions. This is the case for inclusion bodies found in HD which are thought to be important to 'soak up' mutant huntingtin elsewhere in neurons, thereby decreasing neuronal toxicity (Arrasate *et al*, 2004). Similarly, higher order protein conformations seem to sequester the toxic effect of soluble Aβ and Tau (Esparza *et al*, 2018). Cryo-EM has emerged as a powerful technique to further our understanding of protein aggregation in neurodegeneration, by providing detailed structural information of the fibril structures of Aβ42 (Gremer *et al*, 2017) and Tau filaments (Fitzpatrick *et al*, 2017). These studies revealed that Tau oligomerisesation and aggregation into NTFs is likely to be disease-specific (Goedert *et al*, 2019). It will be interesting to examine the composition of these aggregates in conjuction with quantitative proteomics methods to generate more complete structural models.

Interestingly, there seems to be some preferential enrichment of particular ubiquitin chain types in some protein aggregates. For instance, Lewy bodies in PD brains are enriched with K29 and K63-linked ubiquitin chains (Zucchelli *et al*, 2010). Linear (Met-1) and K48-linked ubiquitin were detected in NTFs and PHFs (Nakayama *et al*, 2019). However, while K29 chains were found in NFTs (Nemes *et al*, 2004), a more sensitive quantitative approach revealed that K11, K48 and K63 but not K29 are found in AD or DLB (Dammer *et al*, 2011). It will be interesting to determine whether more complex ubiquitin chain types such as mixed and branched chains can also be found in some of these proteins inclusions. Proteomics studies have been useful for the non-biased determination of the protein composition of these aggregates. For example, Lewy bodies were shown to contain multiple components of the ubiquitin system including E1-conjugating enzyme, the DUBs Otubain 1 and

UCLH1, proteasome subunits, SCF E3 ligases and the E4 ubiquitin ligase UBE4B (Xia *et al*, 2008).

3.2. Crosstalk between Tau, Aβ and ubiquitin signaling

The UPS also plays a role in synaptic plasticity, a vital element of synaptic function, through the ubiquitination of Protein Kinase A (PKA) subunits as well as multiple other synaptic proteins (Hegde *et al*, 1993; Ehlers, 2003). Reduction in NMDA and AMPA receptor expression is associated with AD and elevated Aβ decreases their surface expression (Snyder *et al*, 2005; Hsieh *et al*, 2006). In fact, a recent study showed that Aβ overexpression mediates the ubiquitination of AMPA receptor and its turnover at the plasma membrane, which leads to synaptic weakening (Rodrigues *et al*, 2016).

The underlying mechanisms linking Aβ abnormalities and UPS dysfunction in AD are also becoming clearer. AD patients exhibit decreased proteasomal activity in the hippocampus, a particularly vulnerable area of the brain during the early stages of the disease (Keller *et al*, 2000). The presence of aggregated proteins has been shown to reduce proteasome function and this is likely exacerbated by Tau and Aβ aggregation (Johnston *et al*, 1998). Scanning transmission electron microscopy revealed that Aβ40 binds to the interior of the proteasome and inhibits the chymotrypsin activity of the 20S catalytic core (Gregori *et al*, 1997; 1995). *In vitro* proteasomal assays further confirmed that oligomeric Aβ40-Aβ42 indeed inhibits chymotrypsin but also petidylglutamyl activity (Tseng *et al*, 2008). Expression of the ubiquitin conjugating enzyme E2K (E2-25K/HIP-2) is upregulated in neurons exposed to Aβ, and has also been shown to inhibit proteasomal function through its association with mutant ubiquitin UBB+1 (Song *et al*, 2003). In the case of Tau, aggregated species found within PHFs co-immunoprecipitates with proteasome subunits (Keck *et al*, 2003). In addition, proteasomes found in PHFs also appeared to be less active, and in line with this, aggregated Tau inhibited proteasomal activity in AD brain samples. The E3 ligase CHIP (C-terminus of heat shock protein 70-interacting protein) might contribute to this given that ubiquitination of microtubule-associated Tau increases its aggregation propensity (Petrucelli *et al*, 2004; Shimura *et al*, 2004).

Mechanisms leading to loss of function of components of the proteasome have also been proposed in AD. For example, the proteasome ubiquitin receptor Rpn10 is cleaved by calpain-mediated cleavage following inhibition of the electron transport chain in neurons (Huang *et al*, 2013). Furthermore, the DNA-damage-inducible 1 protein Ddi1, a ubiquitin receptor that aids recruitment of ubiquitinated substrates to the proteasome, is mutated in early-onset AD (Alexander *et al*, 2016). Protein deubiquitination on the proteasome lid is a key step which needs to be tightly coupled to substrate processing and degradation. However, the proteasomal DUB USP14 can release partially ubiquitinated substrates from the proteasome prematurely and prior to their degradation and this reduces proteasome activity (Lee *et al*, 2010). Excitingly, inhibition of USP14 DUB activity was shown to enhance proteasome activity and improve Tau degradation (Lee *et al*, 2015; Boselli *et al*, 2017). In **Table 1** we provide a summary of some of the E3 ubiquitin ligases and deubiquitinases which have been linked with AD.

3.3. Mutant ubiquitin UBB+1

APP and the ubiquitin gene *UBB* have been found mutated by a process called molecular misreading. The resulting mutant protein APP+1 and UBB+1 were found to accumulate in NFTs of frontal and temporal cortices of the hippocampus in AD and Down's Syndrome individuals (Van Leeuwen *et al*, 1998). Molecular misreading is caused by dinucleotide deletion at GAGAGA sequences in mRNA transcripts, resulting in frame shift and the expression of +1 proteins with an aberrant C-terminus. In the case of ubiquitin, this results in an uncleavable 19 amino acid extension at the C-terminus of ubiquitin (UBB+1). Although molecular misreading was first reported in prokaryotic cells, neurons seem particularly prone to such transcriptional error (Evans *et al*, 1994). While low levels of UBB+1 are turned over by the proteasome, its accumulation drives mitochondrial stress and neuronal cell death (van Tijn *et al*, 2007; Fischer *et al*, 2003; De Vrij *et al*, 2001; Tan *et al*, 2007). UBB+1 accumulation seems disease-specific since it is not found in synucleinopathies (Fischer *et al*, 2003). Although UBB+1 does not affect the proteolytic activity of the 20S directly, it inhibits the activity of proteasomal DUBs (Krutauz *et al*, 2014). UBB+1 cannot be used like wild-type ubiquitin to covalently modify proteins due to the absence of the G76 residue. However, it can still be ubiquitinated, primarily with mixed K29/K48-linked polyubiquitin chains although other linkages might also be implicated (Lindsten *et al*, 2002; Chojnacki *et al*, 2016). Interestingly, the toxic potential of the ubiquitinated pools of UBB+1 appears to be different between organisms. UBB+1 ubiquitination with K29 and K48-linked chains is required for full inhibition of proteolysis in mammalian cells but not in yeast (Lindsten *et al*, 2002; Braun *et al*, 2015). The finding that Aβ42 increases the expression of the E2-conjugating enzyme E2-25K/Hip2 is particularly interesting given that E2-25K/Hip2 interacts with UBB+1 and drives apoptosis (Song *et al*, 2003). This study therefore highlights functional links between AD hallmarks and the UPS.

4. Emerging topics in proteostasis and AD

4.1. Mitochondria dysfunction

Mitochondria are the energy generating organelles of all eukaryotic cells by producing ATP molecules through the oxidative phosphorylation pathway. Dysfunction in mitochondria in AD was first suspected through microscopy studies (Saraiva *et al*, 1985). Since then, most if not all hallmarks of AD, including Aβ, Tau and UBB+1, have been associated with mitochondrial dysfunction (Tan *et al*, 2007; Lustbader *et al*, 2004; Rhein *et al*, 2009; Casley *et al*, 2002). Amyloid-β for instance was shown to accumulate in the mitochondrial matrix of neuronal cells from AD transgenic mice (Manczak *et al*, 2006). Mitochondrial homeostasis is maintained by multiple pathways including fission and fusion as well as through the removal of damage mitochondria via mitophagy (Escobar-Henriques & Langer, 2014). Increased mitochondrial fragmentation for instance is observed in AD brains and it is associated with Aβ expression. In fact, Aβ physically interacts with mitochondrial dynamin-related protein 1 (Drp1), a protein involved in mitochondrial fission (Manczak *et al*, 2011). This results in increased mitochondrial fragmentation which affects mitochondrial dynamics and causes synaptic

damage. Ubiquitin signaling is tighly connected to mitochondrial homeostasis. For example, the E3 ubiquitin ligase MITOL/MARCH5 regulates Mitochondrial fission 1 protein (Fis1) and Drp1 levels, which in turn impact on mitochondrial dynamics (Yonashiro *et al*, 2006). Loss of MITOL was shown to preserve mitochondrial function during neuronal stress, including during Aβ-induced stress.

The Ring-Between-Ring (RBR) ligase Parkin is perhaps the best studied mitochondrial E3 ligase identified to date. Parkin and PTEN-induced putative protein kinase 1 (PINK1) form the core components of mitophagy, including in neuronal cells (Ashrafi *et al*, 2014; Vives-Bauza *et al*, 2010; Narendra *et al*, 2008). Mutations in the Parkin gene associated with PD were first identified in 1998 (Kitada *et al*, 1998; Shimura *et al*, 2000; Ye *et al*, 2015; Khandelwal *et al*, 2011). Since then, Parkin mutations have been linked to the pathogenesis of a number of additional brain diseases including AD and multiple sclerosis (Witte *et al*, 2009; Ye *et al*, 2015). PINK1 also appears to be important in AD, since restoring its expression lowers Aβ levels and also reduces mitochondrial and synaptic dysfunction (Du *et al*, 2017). The accumulation of damaged mitochondria seen in AD brains, is a result of inadequate Parkin-mediated mitophagy (Ye *et al*, 2015). Recent structure-function studies have revealed how Parkin mutations found in PD affect its ligase activity and function during mitophagy (Wauer & Komander, 2013). This is also informative for AD, given that PARK2 mutations have also been found in some individuals with sporadic early-onset AD (Barber *et al*, 2017).

Calcium is another major regulator of mitochondrial function which is highly relevant to AD. The atypical Rho GTPases Miro for example anchors mitochondria to motor proteins and regulates mitochondrial trafficking in a calcium-dependent manner (Sheng, 2014). Under conditions that leads to excess intracellular calcium concentration, just as those observed during excitotoxicity, Miro recruits and is ubiquitinated by Parkin, which drives the removal of damaged mitochondria. Under these same conditions, Miro depletion prevents Parkin mitochondrial translocation and this protects neurons from glutamate-induced mitophagy (Safiulina *et al*, 2018). These studies highlight novel crosstalk between calcium signaling, the UPS and mitochondrial function and these are also likely to be relevant in the context of AD.

4.2. ER-Mitochondria-Associated Membranes

The endoplasmic reticulum (ER) has emerged as an important network which is tightly connected to most, if not all, membrane-bound organelles including the plasma membrane (PM), peroxisomes, mitochondria, golgi, lipid droplets and endosomes (Wu *et al*, 2018). Contact sites between the ER and mitochondria, known as ER-Mitochondria-Associated Membranes (ER-MAMs), were first visualized under electron microscopy over 60 years ago (Herrera-Cruz & Simmen, 2017). ER-MAMs regulate organelle dynamics (Friedman *et al*, 2011), autophagy (Hamasaki *et al*, 2013), lipid metabolism and trafficking (Vance, 1991), calcium dynamic (Rizzuto *et al*, 1998; Hirabayashi *et al*, 2017), metabolism (Betz *et al*, 2013), and apoptosis (Simmen *et al*, 2005). At ER-MAMs, ER resident IP3R (inositol 1,4,5-triphosphate receptor) interacts with the outer mitochondrial membrane (OMM) protein VDAC (voltage-dependent anion-selective channel). Both IP3R and VDAC function as

calcium channels, while Grp75 mediates and regulates IP3R-VDAC interactions, thus increasing mitochondrial calcium uptake efficiency (Szabadkai *et al*, 2006). IP3R and VDAC are both substrates of protein ubiquitination, which suggests interesting crosstalks between the ubiquitin system and inter-organelle communication (Sun *et al*, 2012; Sliter *et al*, 2011). For example, the E3 ligase MITOL regulates ER-tethering to the mitochondria, through Mitofusin 2 ubiquitination and degradation (Sugiura *et al*, 2013). ER-MAMs are also highly relevant in AD, with Aβ shown to associate with VDAC1, which enhances its conductance and leads to apoptotic cell death (Smilansky *et al*, 2015). Recent data also indicate that C99, the APP fragment produced by β-secretase-mediated cleavage, increases at ER-MAMs and interferes with mitochondrial function in a cellular model of AD (Pera *et al*, 2017). The other fragment produced from that cleavage event, Aβ, increases the number of ER-MAMs contact sites which raises mitochondrial calcium levels (Hedskog *et al*, 2013). The hyperphosphorylated Tau P301L mutant, a hallmark on FTD, is enriched at ER-MAMs which might further explain the mechanisms underlying Tau-mediated mitochondrial toxicity (David *et al*, 2005). Together, these and other studies strongly implicate ER-MAMs dysregulation in AD and related dementias (Area Gomez *et al*, 2018).

4.3. Necroptosis and AD

Apoptotic cell death is the best studied mode of progammed-cell-death in eukaryotes. It can be triggered by the extrinsic (e.g. Tumour Necrosis Factor-α, Fas Ligand) or intrinsic (e.g. translocation of BAX to the mitochondrial matrix) pathways, which lead to the activation of caspases. In AD, glutamate-induced excitotoxicity mediates neuronal apoptosis. More recently, necroptosis which originally was not believed to be regulated at the molecular level, has emerged as a second mode of programmed-cell-death relevant in AD (Degterev *et al*, 2005; 2008). Both apoptosis and necroptosis are activated by ligand binding of TNFα onto its cognate receptor TNFR1. Necroptosis likely acts as a fail-safe mechanism for cell death since inactivation of apoptosis is a prerequisite for necroptosis induction. While apoptotic cells release their content through the form of apoptotic bodies, necroptotic cells directly leak theirs into the extracellular space. In addition to this phenotypic difference, both cell death pathways are regulated by distinct mechanisms. In contrast to apoptosis, necroptosis does not involve the sequential activation of caspases but rather, it relies on the activation of the ripoptosome, a multi-protein complex which contains serine-threonine kinase receptor-interacting protein kinase 1 and 3 (RIPK1, RIPK3) (Pasparakis & Vandenabeele, 2015). RIPK1/K3 converge to induce the phosphorylation of MLKL (mixed lineage kinase domain-like). Necroptosis culminates in pore formation which is mediated by MLKL, directly and/or indirectly. In the direct model, MLKL phosphorylation leads to exposure of a 4-helical bundle domain which results in its relocalisation and oligomerization at the PM where pore formation occurs (Hildebrand *et al*, 2014). In contrast, the indirect model suggests that MLKL recruits Ca^{2+} channels which increases calcium influx and leads to PM rupture (Cai *et al*, 2014). Recent evidence have reported the upregulation of MLKL and RIPK1 in post-mortem AD brains suggesting that necroptosis is activated

in sporadic AD (Caccamo *et al*, 2017). In support of this, viral-vector mediated expression of MLKL led to more severe cognitive deficits in an AD mouse model, and these effects could be blocked by necrostatin-1, a RIPK1 inhibitor.

Cellular inhibitors of apoptosis 1 and 2 (cIAP1 and 2) are E3 ligases mediating apoptosis. In contrast, much less is known regarding the role of ubiquitin in necroptosis. A recent study reported that expression of HECT E3 ubiquitin ligase SMURF1 is increased upon lipopolysaccharide (LPS)-induced inflammation, and this is accompanied by an increase in the necroptosis marker RIPK1 (Shao *et al*, 2018). Examining the therapeutic potential of RIPK1 and of the ubiquitin system could lead to new and exciting approaches for AD. Encouraging data have shown that the proteasomal inhibitor Carfilzomib prevents ripoptosome complex formation and reduces necroptosis in multiple myeloma cells, although the exact molecular mechanisms remain to be determined (Ali & Mocarski, 2018).

4.4. Targeting the ubiquitin system in AD

The prevailing dogma suggests that maintaining proteostasis throughout life might protect or at least delay age-related disease including AD. Although the protective effect of caloric restriction on health and survival in rhesus monkeys has been controversial, it nevertheless seems to show some cognitive benefits (Mattison *et al*, 2017). Studies in rodents have provided strong evidence to suggest that a decrease in protein synthesis through mTOR inhibition can decrease the toxic effect associated with protein aggregation, while loss of autophagy can cause neurodegeneration in mice (Caccamo *et al*, 2014; Rubinsztein *et al*, 2015; Komatsu *et al*, 2006). Enhancing autophagy and UPS function have been put forward as promising strategies to maintain neuronal health (Myeku & Duff, 2018; Rubinsztein *et al*, 2012). In the context of the UPS, this could be achieved through increasing peptidase activity within the proteasomal core. Alternatively, USP14 inhibition could reduce the premature release of partially deubiquitinated substrates from the proteasome and therefore prevent the accumulation of non-functional proteins (Lee *et al*, 2010). Another strategy could be to increase the recruitment of proteasomal particles to dendritic spines, which are key synaptic contact points where neuronal processes occur (Bingol *et al*, 2010). The stimulation of 26S proteasomal activity has also been achieved through the cAMP-PKA-mediated phosphorylation of proteasomal subunits, such as Rpn6/PSMD11, which enhances the degradation of misfolded proteins (Myeku *et al*, 2016; Lokireddy *et al*, 2015). Cyclic nucleotide phosphodiesterases (PDEs) act as part of a negative feedback loop to dampen the effect of second messengers. PDE inhibition, which prolongs the activation of proteasomal function induced by cAMP, has already shown encouraging results in AD mouse models (Vitolo *et al*, 2002; Smith *et al*, 2009).

Another emerging approach is the targeted degradation of specific molecules by proteolysis targeting chimeric molecules (PROTACs) (Sakamoto *et al*, 2001). These molecules have the capacity to recruit the ubiquitin machinery to target specific proteins for degradation (Zhou *et al*, 2000). PROTACs are hetero-bifunctional molecules, composed of a linker attached to two ligands. One

ligand recruits the E3 ligase while the other recruits the target protein. The recruitment of the target protein to the E3 ligase results in its ubiquitination and subsequent degradation. In theory, this technology has the potential to utilise the endogenous activity of a whole spectrum of E3 ligases in the mammalian genome to degrade a vast array of target proteins. Initially, *in vivo* applications of PROTACs had been problematic due to their poor cellular permeability, but the development of specialised *in vivo* PROTACs containing poly-arginine tags for improved permeability seem to facilitate the degradation of target proteins (Schneekloth *et al*, 2004). Small molecule based PROTACs have also improved cell permeability over their peptide-based counterparts. The first small molecule based PROTAC was composed of the E3 ubiquitin ligase MDM2 ligand Nutlin and a non steroidal androgen receptor ligand, which efficiently degradaded the androgen receptor (Schneekloth *et al*, 2008). Additional PROTACs have since been developed to degrade Cellular Retinoic Acid-Binding Protein 1 (CRABP1) in neuroblastoma cell lines (Itoh *et al*, 2010), bromodomain and extra-terminal (BET) proteins in leukemia (Winter *et al*, 2015) and prostate cancer cells (Raina *et al*, 2016). Excitingly, Arvinas's Androgen Receptor (AR)-PROTAC^{TM} entered the first phase 1 clinical trials for patients with metastatic castration-resistant prostate cancer in 2019. In the context of AD, PROTACs could in theory be developed for the targeted degradation of mutant Tau or of proteases involved in Aβ production. The latter option is perhaps not straightforward given that Aβ also plays a physiological role in neurons. However Tau, Aβ and UBB+1 can impede UPS activity, and this might affect the efficacy of PROTACs in AD (Keller *et al*, 2000). Therefore, an approach that combines enhancing proteasomal activity with PROTACs might be necessary to overcome this limitation. Another mode of targeted protein degradation which could prove useful in AD employs the cytosolic Fc receptor TRIM21 which was recently shown to neutralize misfolded Tau and its associated seeding propensity (McEwan *et al*, 2017). These new technologies, and the ability to target α-synuclein fibrils, have the potential to change the therapeutic landscape in AD over the next few year.

Finally, the recent development of USP7 inhibitors as novel cancer therapies might also be promising in AD (Turnbull *et al*, 2017; Kategaya *et al*, 2017; Pozhidaeva *et al*, 2017; Lamberto *et al*, 2017; Desroses & Altun, 2017). USP7 deubiquitinates and inactivates MDM2 ligase activity which in turns stabilises p53 and enables cancer cells to survive stress conditions. It will be interesting to to see how USP7 inhibitors perform in clinical trials and also to explore DUB-based therapies in AD (**Table 1**). For instance, USP14 inhibition accelerates the degradation of mutant Tau (Boselli *et al*, 2017), while loss of USP8 DUB activity leads to the lysosomal degradation of BACE1 which in turn reduces Aβ production (Yeates & Tesco, 2016). Importantly, DUBs and E3s likely have multiple substrates and the specificity of the mechanisms targeted will need to be closely monitored (Huang & Dixit, 2016).

5. Concluding remarks

Despite the discovery of protein inclusions in the brain of patients with AD over a century ago, there

589 is still no cure available. GWAS studies have been useful to identify biomarkers in familial cases, but
590 the molecular mechanisms that initiate and drive AD remain elusive. Determining the properties and
591 function of Aβ and Tau in normal brain homeostasis and AD has been a focus for many years and it
592 now appears that soluble intermediate oligomers, rather than protein aggregates may well be the
593 toxic species. AD and other brain proteinopathies have been associated with loss of proteostasis
594 and the decrease in proteasomal function and autophagy contribute to this process. The brain seems
595 to be particularly reliant on these pathways to maintain homeostasis, which likely reflects the inability
596 of neurons to divide or replenish following injury. In this chapter, we have summarised some the
597 evidence linking dysregulation in ubiquitin signaling and AD, including crosstalk between Tau, Aβ
598 and the UPS. We have also highlighted new ubiquitin-dependent mechanisms and in particular how
599 changes in deubiquitinases and E3 ubiquitin ligases activity impact on synaptic and cognitive
600 function. Neuronal necroptosis, mitochondrial homeostasis and calcium signaling at ER-MAMs have
601 all emerged as exciting new fields of research relevant for AD. It will be interesting to determine how
602 the writers (i.e. E1, E2, E3s), readers (i.e. UBDs) and erasers (i.e. DUBs) of the ubiquitin code impact
603 on these mechanisms during healthy and pathological brain aging. Technological advances
604 including cryo-EM, quantitative proteomics, single molecule microscopy as well as improved *in vitro*
605 and *in vivo* AD models will be instrumental in further defining how such proteinopathies initiate and
606 develop. This fundamental knowledge should then feed into drug development with the aim to open
607 new therapeutic avenues for AD.

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614 **Table 1. E3 ligases and DUBs in AD**
615

Protein name	Protein Family	Evidence/mechanisms	References
E3 ubiquitin ligases			
Parkin	RBR	Parkin is found in neurofibrillary tangles.	(Nemes *et al*, 2004)
		Parkin depletion during the course of AD in hAPP neurons and AD patient brains.	(Ye *et al*, 2015)
		Mitophagy inhibits Aβ and Tau pathology.	(Fang *et al*, 2019)
APC/c	RING	Loss of function leads to learning and memory impairment phenotypes in mice.	(Kuczera *et al*, 2011)
		Increase activity through elevated expression of the activator Cdh1 prevents cell cycle re-entry of neurons via suppression of cyclin B.	(Aulia & Tang, 2006)
BRCA1	RING	Reduced BRCA1 levels found in the brain of AD patients.	(Suberbielle *et al*, 2015)
CHIP/ STUB1	RING	Ubiquitin-dependent degradation of phosphorylated Tau.	(Shimura *et al*, 2004; Petrucelli *et al*, 2004)
		Binds to and functions in a complex with $CRL4^{CRBN}$ to ubiquitinate APP via the APP cytosolic region.	(Del Prete *et al*, 2016)
Dactylidin/RNF146	RING	RNF146 is upregulated in AD brains.	(Rotz *et al*, 2005)
GRN/ Progranulin	RING	GRN mutations are associated with FTD-U.	(Baker *et al*, 2006; Cruts *et al*, 2006; Gass *et al*, 2006; Galimberti *et al*, 2010)
HRD1/ synoviolin	RING	HRD1 is expressed in neurons and reactive astrocytes and might be associated with the ubiquitin-dependent degradation of hyperphosphorylated Tau.	(Hou *et al*, 2006)
		HRD1 is inhibited by soluble Tau accumulation which leads to activation of the unfolded protein response.	(Abisambra *et al*, 2013)
		Loss of HRD1 leads to APP and Aβ accumulation.	(Kaneko *et al*, 2010)
		Loss of function leads to Aβ generation.	(Maeda *et al*, 2009; Kaneko *et al*, 2010; Tanabe *et al*, 2012)
MARCH7	RING	MARCH7 ubiquitinates 4-repeat (4R) Tau and impairs microtubule binding.	(Flach *et al*, 2014)
MGRN1	RING	Involved in the maturation and trafficking of APP.	(Benvegnù *et al*, 2017)
MYLIP/ IDOL	RING	Targets LDLR-dependent APOE and Aβ for clearance. Candidate therapeutic target for AD.	(Choi *et al*, 2015)
TRAF6	RING	Prevents Aβ-induced neuronal death through ubiquitin-dependent degradation of p75 neurotrophin receptor.	(Geetha *et al*, 2012)
ZNRF1	RING	ZNRF1 Promotes neurodegeneration through the degradation of Akt via the UPS.	(Wakatsuki *et al*, 2011)
FBXO2	SCF	Regulates APP levels and processing in the brain.	(Atkin *et al*, 2014)
CCNF	SCF	Abnormal ubiquitination and accumulation of TDP-43 and SCF substrates in amyotrophic lateral sclerosis and FTD.	(Williams *et al*, 2016)
SLIMB	SCF	SLIMB mediates the ubiquitin dependent UPS degradation of active and phosphorylated PAR-1. This impacts on Tau-mediated postsynaptic toxicity of Aβ.	(Lee *et al*, 2012)

TRIM11	TRIM	TRIM11 targets humanin, a neuroprotective peptide that suppresses AD-related neurotoxicity, to the UPS.	(Niikura *et al*, 2003)
TRIM21	TRIM	TRIM21 mediates the UPS-mediated degradation of Tau and this inhibits seeding aggregation.	(McEwan *et al*, 2017)
TRIM28	TRIM	Stabilises Tau and α-synuclein nuclear accumulation.	(Rousseaux *et al*, 2016; 2018)
TRIM32/37	TRIM	Altered expression in AD brains.	(Yokota *et al*, 2006)
NEDD4	HECT	Regulates AMPR ubiquitination, turnover and trafficking.	(Lin *et al*, 2011; Hou *et al*, 2011)
		Aβ increases turnover of AMP receptor through NEDD4-dependent ubiquitination and lysosomal degradation	(Zhang *et al*, 2018)
		Aβ-induced synaptic alterations correlate with AMPAR ubiquitination by NEDD4.	(Rodrigues *et al*, 2016)
		Ubiquitination of GluA1 by NEDD4-1 is activated by neuronal signaling and leads to GluA1 endocytosis and lysosomal trafficking.	(Schwarz *et al*, 2010)
NDFIP1	NEDD4 adaptor	NDFIP is implicated in the degradation of divalent metal transporter 1 (DMT1). Loss of NDFIP leads to increased iron dyshomeostasis and Aβ production.	(Tian *et al*, 2018)
HUWE1A	HECT	Proteome-wide study identifies lower HUWE1A protein levels in AD brain.	(Ho Kim *et al*, 2015)
UBE3A	HECT	UBE3A-deficient AD mice have reduced Aβ levels and plaque formation and show accelerated cognitive and motor deficits compare with AD mice.	(Singh *et al*, 2017)
UBR5	HECT	Novel loci associated with disease progression in subjects with mild cognitive impairment in AD.	(Hu *et al*, 2011)
Deubiquitinases			
OTUB1	OTU	OTUB1 deubiquitinates Tau *in vivo* and *in vitro* and regulates Tau oligomeric forms.	(Wang *et al*, 2017)
USP46	USP	Aβ induces internalization and subsequent ubiquitination and lysosomal degradation through NEDD4 upregulation and downregulation of USP46.	(Zhang *et al*, 2018; Huo *et al*, 2015)
USP14	USP	USP14 inhibition accelerates degradation of WT Tau as well as pathological Tau mutants P301L and P301S, and the variant A152T.	(Boselli *et al*, 2017)
USP8	USP	Regulates the ubiquitination, trafficking and lysosomal degradation of BACE1.	(Yeates & Tesco, 2016)
		Regulates α-synuclein clearance in Lewy Bodies.	(Alexopoulou *et al*, 2016)
UCHL1	UCH	Elevated protein levels in AD patients, decreased mRNA and protein level in DLB.	(Ohrfelt *et al*, 2016; Barrachina *et al*, 2006)
		Controversial genetic association between UCHL1 polymorphism and sporadic AD.	(Xue & Jia, 2006; Forero *et al*, 2006; Shibata *et al*, 2012)
		DUB activity required for normal synaptic and cognitive function.	(Gong *et al*, 2006)
		Overexpression accelerates APP lysosomal degradation and reduces Aβ production.	(Zhang *et al*, 2014)
		Aβ affects BDNF retrograde trafficking through inhibition of UCHL1 activity.	(Poon *et al*, 2013)
		Inhibition of UCHL1 DUB activity decreases Tau binding to microtubules and increases its phosphorylation.	(Xie *et al*, 2016)

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