The Role of Ubiquitin-Protein Ligases in Neurodegenerative Disease

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Key Words

Ubiquitylation · Ubiquitin-protein ligase · Skp1-Cdc53/cullin-F-box complexes · RING domain · HECT domain · Neurodegeneration · Inclusions · Aggresomes

Abstract

Alzheimer's disease and Parkinson's disease are the most common neurodegenerative conditions associated with the ageing process. The pathology of these and other neurodegenerative disorders, including polyglutamine diseases, is characterised by the presence of inclusion bodies in brain tissue of affected patients. In general, these inclusion bodies consist of insoluble, unfolded proteins that are commonly tagged with the small protein, ubiquitin. Covalent tagging of proteins with chains of ubiquitin generally targets them for degradation. Indeed, the ubiquitin/proteasome system (UPS) is the major route through which intracellular proteolysis is regulated. This strongly implicates the UPS in these disease-associated inclusions, either due to malfunction (of specific UPS components) or overload of the system (due to aggregation of unfolded/mutant proteins), resulting in subsequent cellular toxicity. Protein targeting for degradation is a highly regulated process. It relies on transfer of ubiquitin molecules to the target protein via an enzyme cascade and specific recognition of a substrate pro-

tein by ubiquitin-protein ligases (E3s). Recent advances in our knowledge gained from the Human Genome Mapping Project have revealed the presence of potentially hundreds of E3s within the human genome. The discovery that parkin, mutations in which are found in at least 50% of patients with autosomal recessive juvenile parkinsonism, is an E3 further highlights the importance of the UPS in neurological disease. To date, parkin is the only E3 confirmed to have a direct causal role in neurodegenerative disorders. However, a number of other (putative) E3s have now been identified that may cause disease directly or interact with neurological disease-associated proteins. Many of these are either lost or mutated in a given disease or fail to process disease-associated mutant proteins correctly. In this review, we will discuss the role(s) of E3s in neurodegenerative disorders.

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Introduction

Neurodegenerative disorders such as Alzheimer's (AD), Parkinson's (PD) and Huntington's (HD) disease and prion disorders are characterised by a selective and symmetric loss of neurons in specific regions of the brain. The result is disruption to motor, sensory or cognitive systems, causing such severe disability that premature death of a sufferer is an inevitable consequence. The majority of

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these diseases affect patients later in life. There is currently no cure or long-term treatment for these progressive diseases. Even where there are therapies, such as the administration of levodopa or other dopamine agonists in PD, these are often only effective in the short term. Consequently, given that in the UK the number of people over 60 years old will soon exceed those under 16 years old [1], it is essential that we elucidate the fundamental processes underlying these disorders in order to develop novel therapeutic agents to help an ageing population.

The cause(s) of sporadic neurodegenerative disease remain(s) elusive. A complex mixture of underlying genetic polymorphisms, possibly in genes associated with hereditary forms of the disease, and/or exposure to environmental stresses may make an individual particularly vulnerable.

The pathological hallmark of many neurodegenerative diseases is the presence of ubiquitin-positive, intra- or extracellular inclusion bodies in affected regions of the brain [2–4]. However, it remains uncertain whether the toxicity associated with their presence or formation is the result of the presence of the inclusion body per se or a loss of normal protein function that results from its sequestration into an inclusion. Moreover, inclusions may occur as a result of attempts by neurons to protect themselves from toxic proteins. Inclusion models have been developed both in mice and cell culture systems, which recapture many of the properties of these endogenous inclusions [5–15]. These are generally based upon the (over-)expression of (mutated) core proteins associated with these inclusions that are also found in rare inherited cases of the diseases, such as β -amyloid (A β) in AD or α -synuclein in PD.

In general, these inclusions consist of insoluble, unfolded, ubiquitylated polypeptides that fail to be degraded by the 26S proteasome. Their apparent stability may, in part, be due to decreased levels of 26S proteasomal activity, which is associated with increasing age [16]. Given the pathological presentation of these conditions, it is perhaps not surprising that an increasing number of proteins associated with the ubiquitin-proteasome system (UPS) are now known to play either a direct or indirect role in familial forms of disease [for recent reviews, see ref. 17–21].

Ubiquitin-protein ligases (E3s) are of particular importance as they determine the targeting specificity of the ubiquitin system. Such molecules have been found to be mutated, absent or malfunction in some hereditary forms of neurodegenerative disorders. These include parkin, dorfins and the recently identified malin. Of all the proteins discussed herein, to date, parkin (and possibly

UCH-L1) is the only one confirmed to have both E3 activity and a direct causal role in neurodegenerative disease. However, a number of others, in particular malin, mutations in which are associated with Lafora disease, are likely to play either direct, or indirect, roles in the aetiology of their associated disorders. The focus of this review will be E3s that may have a functional role in human neurodegenerative disorders.

Aberrant Protein Accumulation: Refold or Degrade?

Regulating the level of a functionally active protein is a highly complex and tightly regulated process. A fine balance between its rate of synthesis and degradation, which may be dependent on post-translational modification, must be maintained. Additional complicating factors may include the availability of one or more interacting proteins and its intracellular location. Normally, newly synthesised polypeptides emerge from the ribosome to be met by molecular chaperones that ensure their appropriate folding and intracellular localisation, whilst preventing their aggregation [22, 23]. Misfolded 'escapees' are rapidly targeted for degradation via the UPS [24].

UPS-mediated post-translational modification and degradation of proteins is essential for many fundamental cellular functions that include cell cycling, DNA repair, cell signalling, gene transcription and apoptosis. Indeed, the UPS is now recognised to be the major route by which proteins are specifically selected for spatiotemporal degradation in eukaryotic cells [for recent reviews, see ref. 25–30].

The covalent attachment of the 76-amino acid residue protein, ubiquitin, to an ϵ -amino group of a lysine residue in a target protein involves an energy-dependent, four-step pathway (fig. 1). Ubiquitin is transferred from an ubiquitin-activating enzyme (E1), to an ubiquitin-conjugating enzyme (E2) and then to the target protein. This latter step is facilitated by an E3. Multiple rounds of ubiquitylation via attachment of additional ubiquitin moieties (generally through ubiquitin Lys48) may then occur leading to the generation of polyubiquitin chains. These polyubiquitylated conjugates (at least 4 ubiquitin molecules must be attached) are then selected for degradation by the 26S proteasome. During this process, ubiquitin is removed and the target protein degraded into small peptides. These latter molecules can be hydrolysed further by other cellular peptidases or used for antigen presentation [31, 32].

The requirement for the addition of multiple ubiquitin molecules may be part of a proof-reading mechanism, as a large number of de-ubiquitinating enzymes (DUBs) are also found within cells [33–35]. This latter group of enzymes may remove ubiquitin moieties before a sufficiently long ubiquitin chain has been synthesized to activate proteasomal destruction. Alternatively, they may also prevent the synthesis of chains of such a length that they promote unwanted aggregation of proteins.

More recently, monoubiquitylation of proteins has been demonstrated to have major functional roles that are apparently independent of degradation, in particular, regulation of endocytosis of cell surface receptors, DNA repair mechanisms and transcription regulation [for a recent review, see ref. 36].

E3s: Enzymes and Scaffolds

The selectivity of the UPS essentially lies in the specificity of interaction between an E3 and a target protein. In some cases, the need for substrate post-translational modification such as phosphorylation permits an E3 to selectively target more than one substrate within a given cell [37, 38]. Hence, the initial trigger for substrate selection will be independent of the ligase but be dependent on the activity of a kinase.

Two major classes of E3 have been identified. These are members of the E6-AP-related family of ‘HECT’ (homologous to the E6-AP C terminus) domain E3s [39] and the potentially much larger ‘RING (really interesting new gene) finger domain’ types [40–42]. These latter types of E3s do not appear to have a direct catalytic role in protein ubiquitylation but act as scaffolds or ‘facilitators of interaction’. By contrast, HECT domain family members directly catalyse the final attachment of ubiquitin to substrate proteins through their approximately 350 amino acid C-terminal HECT domains [39].

One of the first E3s to be identified and characterised was E6-AP1 (E6-associated protein 1) [43]. Subsequently, many other proteins characterised by the HECT domain have been identified [39, 44–47]. HECT type E3s function in a similar manner to E1 and E2s containing a defining Cys residue in the HECT domain. The thiol group of the Cys residue forms a thioester bond with the carboxy group of the glycine residues found at the C terminus of ubiquitin prior to its transfer to the target protein.

However, E3s characterised by a RING finger domain are likely to represent the largest group of ligases. In fact, the RING domain is the fourth most common protein motif in the human genome, indicating that although unlikely that all of these will display E3 activity, many

Fig. 1. Protein-mediated degradation via the ubiquitin-proteasome pathway. Ubiquitylation occurs in a step-wise manner. (1) Energy-dependent thioester bond formation between the C-terminal glycine residue of ubiquitin (Ub) and a thiol group located within an ubiquitin-activating enzyme (E1). (2) Ubiquitin is then transferred to an ubiquitin-conjugating enzyme (E2) via transthioesterification. (3) E3s then facilitate ubiquitylation of the target protein (Pr). E3s may be large multi-subunit complexes (left-hand side) or single proteins (right-hand side). Multiple ubiquitin molecules may then be covalently attached to one of several lysine residues found within a pre-attached ubiquitin to form a polyubiquitylated protein. (4) Chains of at least 4 ubiquitin molecules are required for recognition by the 26S proteasome. Polyubiquitylated proteins not selected for immediate 26S proteasomal-mediated, ATP-dependent, degradation may be deubiquitylated by one of many DUBs found within cells.

more E3s of this type are yet to be elucidated [48]. These E3s are involved in regulating diverse cellular functions including roles in apoptosis, cell cycle control and tumour suppressor function [49, 50]. They can act either alone [50–54] or as a component of a multiprotein E3 ligase complex such as an Skp1-Cdc53/cullin-F-box (SCF) complex [55, 56], the anaphase-promoting complex [57, 58] or the von Hippel-Lindau tumour suppressor protein complex [59, 60].

The RING finger domain is a Cys/His-rich, zinc-chelating domain that promotes both protein to protein and protein to DNA interactions [61]. It appears that the RING structure is essential for the E2:E3 interaction and, consequently, the E3 activity of the protein [50–54]. Importantly, although many proteins characterised by a RING finger motif possess E3 activity in vitro, there is no evidence to suggest that all RING finger proteins behave as E3s under normal physiological conditions. Indeed, the promyelocytic leukemia tumour suppressor protein,

which is a RING protein, inhibits mRNA translation by initiation factor eIF4E by mechanisms that do not involve the UPS [62].

Recently, a putative third class of E3s has been identified and characterised, the U-box ligases [63–65]. These proteins appear to act in a similar manner to RING finger proteins. However, they lack the characteristic Cys and His residues that define the RING finger domain structure. Nevertheless, due to their structural similarity to the RING motif, they may also be classified as a subclass of RING finger type E3s [66].

Of note, many HECT and RING finger domain E3s ‘autoubiquitylate’ in *in vitro* and in eukaryotic cell overexpression systems [67–69]. Although this may simply be an *in vitro* artefact brought about by high levels of expression of a given protein, autoubiquitylation may represent a gain-of-function and protective cellular mechanism, by which certain types of proteins including RING finger proteins are programmed to self-destruct to prevent their aggregation when overexpressed. The latter proposal may be particularly relevant in neurological disorders associated with ageing where reduced proteasomal activity may cause increased levels of E3 autoubiquitylation as well as stabilisation of substrate proteins.

Pathological Inclusions and Aggresomes

The pathological hallmark of many neurodegenerative diseases is the presence of insoluble intra- or extracellular inclusion bodies in affected regions of the brain. The major constituents of these inclusions are misfolded proteins. Their aggregation into proteinaceous inclusions is perhaps surprising given that there are cellular processes present designed to prevent such events. Moreover, what is also puzzling is the observation that these inclusions are found almost exclusively within certain subgroups of neurons in specific regions of the brain regardless of the widespread tissue distribution of the core proteins.

The classic causes for protein misfolding, which lead to a loss of function, are missense mutations, protein modifications or post-translational damage, or expansion of amino acid repeats as is observed in polyglutamine disorders. Misfolded proteins may be ‘rescued’ via chaperones and co-chaperones as has been observed for ataxin-1, parkin-associated endothelin-receptor-like receptor (Pael-R) and α -synuclein [70–72]. However, should these chaperone-mediated pathways or the UPS fail, misfolded proteins that are prone to aggregation may form insoluble inclusions (fig. 2). These inclusion bodies are usually characterised by the presence of aggregates of one major ‘core’ protein, or peptide, and a series of other associated proteins.

The latter components often include chaperones such as Hsp70, Hsp27 and α B-crystallin [73–76]; UPS components including parkin, proteasomal subunits and ubiquitin [2, 77–79], and cytoskeletal elements such as tau, tubulin-associated proteins and neurofilaments [80–83].

Overexpression of a number of neurodegenerative disease-associated proteins including presenilin 1 (PS1), parkin and huntingtin (associated with AD, PD and HD, respectively) in proteasome-inhibited mammalian cells leads to inclusion formation [5, 10, 12, 15]. Moreover, disease-associated mutant proteins readily form aggregates without the need for proteasome inhibition [10, 15, 84, 85]. These inclusions have been characterised as ‘aggresomes’ [5, 10, 86–88]. It is postulated that endogenous aggresomes occur when increased levels of substrate expression exceed the capacity of the proteasome. Indeed, McNaught et al. [76] have recently reported that the proteinaceous pathological hallmark of PD, the Lewy body, may represent a specialised aggresome-related inclusion specific to dopaminergic neurons.

UPS in Neurodevelopment and Neurodegeneration

Although the aim of this review was to focus on the role of E3s in neurodegeneration, the functions of the UPS in neurological conditions is by no means restricted to problems of later life. Indeed the function of several UPS components has been implicated in normal eukaryotic neurodevelopment. A mutation in the *Drosophila* *Bendless* gene, which encodes an E2, impairs axonal guidance [89, 90]. Null mutants of *Ariadne-1*, a *Drosophila* RING finger domain E3, result in embryonic lethality at the pupal stage, with occasional adult survivors displaying severe disruption of the central nervous system [91]. Furthermore, targeted neuronal overexpression of *Fat facets*, a DUB, results in abnormal synaptogenesis and impaired synaptic function in *Drosophila* [92]. In humans, loss of activity of the imprinted gene coding for the HECT E3, E6-AP1, leads to Angelman syndrome, a condition associated with severe motor and intellectual retardation and ataxia [93, 94].

E3s in Neurodegenerative Disorders

There has been a rapid explosion in our knowledge of the UPS and its many cellular functions over the last 5 years. One of the major discoveries was the characterisa-

Fig. 2. Inclusion bodies are a common occurrence in most neurodegenerative diseases. **a** Correct folding of newly synthesised proteins is mediated by the chaperone system to produce functionally active proteins (post-translational modifications may also be required). Misfolded proteins are either unfolded and refolded by chaperones or targeted to the UPS for degradation. **b** In many inherited neurodegenerative conditions, most of the newly synthesised mutant protein (mutation shown as a grey sphere) may be rapidly targeted to the UPS via ubiquitylation. However, some may be correctly folded, with few displaying functional activity. Ubiquitylated, misfolded mutant proteins and/or poorly ubiquitylated folded protein may not be easily recognised by the proteasome causing a build-up of ubiquitylated species. These 'pre-inclusions' may be highly toxic to the cell and cause rapid cell death. Protein aggregates that reach the proteasome may cause blockage of the degradation machinery preventing proteolysis of other UPS targets, causing greater accumulation of proteins and aggresome formation. These inclusions will eventually cause severe impairment of proteasome activity resulting in increased cellular toxicity and eventual cell death. In sporadic disease, a slowing down of proteasomal activity with age may lead to an increase in aggregated proteins causing inclusion formation. In either scenario, exposure to additional environmental stresses may make a cell more susceptible to inclusion formation.

Table 1. E3s associated with neurodegenerative disorders

Disease	E3(s) implicated in disease	Type of E3
Alzheimer's disease	SCF ^{hCdc4} TRIM11	multicomponent RING
Parkinson's disease	parkin ¹ CHIP UCH-L1 ¹ dorfin	RIR U-box novel, requires dimerisation RIR
Amyotrophic lateral sclerosis	dorfin	RIR
Huntington's disease	RNF2 RNF24 HIP1-8 HSD-4	RING RING RING RING
Spinobulbar muscular atrophy	SNURF/RNF4 ARA54 ARNIP	RING RIR RING
Ataxia, SCA1	E6-AP	HECT
Dentatorubral-pallidoluysian atrophy	AIP2 AIP4/ITCH AIP5	HECT HECT HECT
Prion diseases	mahogunin	RING
Lafora disease	malin ¹	RING

E3s which have been either directly linked to a neurodegenerative disease or are known to interact with disease-associated proteins.

¹ Mutations/deletions in the genes encoding these proteins have been directly linked to some familial forms of disease.

tion of the RING finger as a (multi-)protein domain capable of E3 activity. Subsequently, hundreds of proteins have been identified within the protein databases that may have ligase activity. Many of these proteins, and those of the E3 family containing an HECT domain, are now being identified as proteins that are either directly responsible for, or associated with specific neurodegenerative disorders (table 1). Our current understanding of the role of the E3 in major neurodegenerative disease is summarised below.

Alzheimer's Disease

Dr. Alois Alzheimer first described AD nearly a century ago in 1906. Despite its high incidence rate (5–10% of the population over 65 years are affected, with a preva-

lence rate increasing to 50% of those over 85 years), and much research, inadequate understanding of the condition means there is still no curative treatment or prevention strategy available. AD affects the central nervous system resulting in loss of memory, declining cognitive function and ultimately decreased physical function. The disease is characterised by the accumulation of extracellular amyloid plaques and cytoplasmic paired helical filaments made up of neurofibrillary tangles [95, 96]. The main constituent of the former is a product (A β) of the processing of the amyloid precursor protein (APP), whereas that of the latter are phosphorylated forms of the microtubule-associated protein, tau. Both types of inclusion are ubiquitin positive [2, 97].

Up to 10% of AD cases are due to inherited forms of the disease. To date, 3 genetic loci have been associated with familial AD: *APP* on chromosome 21, *PS1* on chromosome 14 and *presenilin 2* (*PS2*) on chromosome 1 [98–100]. All 3 mutant genes can increase A β deposits and subsequent plaque formation. PS1 and PS2 are likely to be components of the multi-protein γ -secretases required for proteolytic cleavage of APP to form A β [101, 102]. Presenilins are degraded via the UPS [103, 104], by an E3 SCF complex containing the F-box protein FBXW7 (Cdc4 in *Saccharomyces cerevisiae*, SEL-10 in *Caenorhabditis elegans*) [105]. Co-expression of FBXW7 and APP causes increased presenilin ubiquitylation and elevated A β production. Whether this is a result of ubiquitylation of full length presenilins or the enzymatically active fragments thereof remains controversial [104, 105]. Additionally, the nature of the components of the PS1 SCF^{FBXW7} complex and the detailed mechanisms that result in PS1 ubiquitylation remain to be resolved. Moreover, it also needs to be established how this activity results in increased levels of A β .

Humanin is a neuroprotective, 24-amino acid polypeptide that is capable of specifically suppressing AD-related neurotoxicity caused by the presence of PS1 and APP mutants [106, 107]. Elevated levels of humanin are found in brain tissue of AD patients relative to age-matched controls [108]. The mechanisms behind the protective effects of humanin remain unclear. Recent studies have indicated that it can prevent Bax activation, thereby suppressing mitochondrial stress-induced apoptosis [109]. Furthermore, humanin levels can be down-regulated by the RING E3, TRIM11 [110]. Therefore, humanin presents a possible therapeutic target for controlling AD. Slowing its rate of degradation by TRIM11 may maximise this peptides' therapeutic potential.

Parkinson's Disease

PD is the second most common neurodegenerative disease. It is characterised by the loss of dopaminergic neurons in the substantia nigra of the midbrain and the presence of ubiquitin-positive proteinaceous cytoplasmic inclusions, Lewy bodies, in many of the remaining neurons [2, 111]. The majority of PD cases are apparently sporadic, with only 5–10% of all cases being inherited [112]. To date, genetic studies have identified mutations in the genes that code for α -synuclein, parkin, UCH-L1, DJ-1 and NR4A2 in families with PD [113–117]. The defective gene(s) at other candidate loci remain(s) to be identified [118, 119]. Although the molecular changes underlying the initiation and progression of sporadic disease are largely unclear, there is now strong evidence that implicates abnormal processing of a variety of cellular proteins via the UPS in PD development. In fact, in the familial forms of PD, α -synuclein, parkin, UCH-L1 and DJ-1 are associated with the UPS, parkin and UCH-L1 as components of the system, modified and/or mutant α -synuclein and DJ-1 as targets [120–122].

Parkin mutations account for about 50% of cases of autosomal recessive juvenile parkinsonism (AR-JP) [123] and a small number of apparently sporadic cases of PD [124–126]. Unlike sporadic PD, Lewy body pathology is apparently absent in brain tissue of AR-JP patients [77, 127]. Parkin is a RING type E3 [54]. Two RING domains are separated by an additional Cys/His-rich region termed the 'in between RING finger' (IBR) domain [128], a domain which is found in a number of other proteins including dorfin, HHARI and H7-AP1/Parc [69, 129]. Disease-associated mutations within the RING-IBR-RING domain of parkin inhibit interaction with the E2s UbcH7 or UbcH8 thereby inhibiting its E3 activity [54, 130, 131]. Consequently, the failure of parkin to ubiquitylate target proteins may lead to their accumulation. In turn, this may lead to the formation of aggregates that are toxic to neurons of the substantia nigra.

To date, 9 targets of parkin-mediated degradation have been identified; CDCrel-1 [131], Pael-R [132], synphilin-1 [6], α -synuclein [121] and/or a glycosylated form of α -synuclein, termed α Sp22 [120], cyclin E [133], α/β tubulin [134], the p38 subunit of the aminoacyl-tRNA synthase complex (p38) [135], SEPT5_v2 [136] and synaptotagmin XI [137]. The significance of parkin-targeted ubiquitylation of these proteins with respect to PD remains largely unclear. Whereas some are found in Lewy bodies, others accumulate in the brains of AR-JP patients [121, 132, 135, 138, 139]. Furthermore, proteins such as α -synuclein

and Pael-R are known to be natively unfolded, prone to aggregation and cause toxicity in neuronal cells [71, 132, 140–142]. Moreover, mutant (but not wild-type) α -synuclein has been reported to inhibit proteasome activity [9, 141, 143]. Interestingly, parkin is capable of suppressing the neurotoxicity of both α -synuclein and Pael-R [141, 142]. This indicates a role for parkin in both sporadic and hereditary forms of PD. In sporadic disease, parkin may become sequestered into Lewy bodies by target proteins including (mutant) α -synuclein and Pael-R. In parkin-associated disease, lack of E3 activity that results in increased substrate levels may lead to the formation of highly toxic protein aggregates. The potency of these toxic aggregates may lead to such a rapid cell death that the remaining neurons do not present aggresome-related Lewy bodies.

Additional E3s may have a significant role in PD. The U-box E3, CHIP, interacts with, and enhances the ubiquitylation of, the Pael-R by parkin [71]. Additionally, dorfin and the RING E3 Siah-1 are both capable of targeting synphilin-1 for degradation [144, 145]. Indeed, Siah-1-mediated ubiquitylation of synphilin-1 was more efficient than that of parkin [145]. Interestingly, with respect to parkin specificity, neither dorfin nor Siah-1 can directly ubiquitylate α -synuclein.

Mutations in the DUB UCH-L1, which drastically reduce its enzymatic activity, cause autosomal dominant PD in a German kindred [115]. However, a rather surprising observation was that UCH-L1 also possesses E3 activity towards α -synuclein in vitro [146]. This ATP-independent process required UCH-L1 dimerisation. An S18Y polymorphism, which is associated with decreased risk of PD, displayed reduced E3 activity [146]. Furthermore, UCH-L1 E3 activity towards α -synuclein generated Lys63 polyubiquitin chains. Taken together, Liu et al. [146] suggested that UCH-L1 may possess both a beneficial hydrolase activity and a dimerisation-dependent ligase activity, which may be pathologically detrimental. It will now be important to confirm the dual activities of UCH-L1 in vivo.

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disorder. It begins in the fifth or sixth decade of life. Death usually then occurs within 5 years. ALS is characterised by a loss of motor neurons in the cerebral cortex, brain stem and spinal cord. Prior to cell death, neurons of ALS patients contain ubiquitin-positive

hyaline and skein-like inclusions [147–149]. Although the majority of ALS cases are sporadic, 10% of patients have familial disease. Autosomal dominant mutations in the *SOD1* gene account for up to 20% of these inherited cases [150]. *SOD1* encodes the superoxide dismutase 1 protein that is involved in free radical scavenging. Similar to other neurodegenerative-associated proteins, mutant *SOD1* has a high propensity to misfold and aggregate [reviewed in ref. 151]. Whilst the precise mechanisms underlying *SOD1*-related disease remain unclear, there is an increasing body of evidence indicating that mutant *SOD1* causes mitochondrial dysfunction and inhibition of the proteasome [152–155]. Recent immunohistochemical analysis revealed that the RING-IBR-RING protein, dorf, localised within the inclusions of ALS patients and interacted with mutant, but not wild-type *SOD1* [13, 149]. Furthermore, Niwa et al. [13] used neuronal cell culture systems to demonstrate that dorf displayed protective properties against mutant *SOD1*-mediated toxicity. It targeted mutant *SOD1* for 26S proteasomal degradation, thereby reducing the number of inclusion bodies [13]. Hence, regulation of dorf E3 activity may be beneficial in the treatment of ALS.

Polyglutamine Disorders

A number of neurodegenerative conditions caused by expanded polyglutamine (polyQ)-encoding CAG repeats have been identified. All are characterised by a toxic gain of function of the disease-associated expanded polyQ protein and the development of ubiquitin-positive neuronal intracellular inclusions and eventual cell death of a subset of neurons [156, 157]. Interestingly, overexpression of parkin reduced the number of inclusions and cytotoxicity observed in cells expressing an expanded polyQ repeat [158]. However, these cell culture conditions represented somewhat artificial conditions. Therefore, it is now essential to determine whether such observations can be replicated in transgenic animals. If so, these data would suggest that modulation of parkin activity would represent a therapeutic option for treatment of at least some of these conditions.

Huntington's Disease

HD is the most common and best characterised of the polyQ disorders. This autosomal dominant disease presents with progressive, selective loss of striatal neurons associated with choreic movements and dementia. The disease is associated with increases in the length of a CAG

triplet repeat present in the *huntingtin* gene located on chromosome 4p16.3. Normal subjects contain between 11 and 34 polyQ repeats within *huntingtin*. By contrast, HD patients generally have more than 40 [159]. Pathologically, affected neurons contain intranuclear ubiquitylated inclusions [160, 161]. Moreover, huntingtin is also found to be ubiquitylated in patients, transgenic mice and cell culture systems [7, 10, 160–162]. The function of huntingtin is unknown. A range of functions that include roles in vesicle trafficking, signal transduction and transcription have been proposed [for a recent review, see ref. 163].

Many groups have utilised the yeast-2-hybrid system in an attempt to identify huntingtin-interacting proteins. One such protein was the E2, E2-25K (HIP-2) [162]. However, its interaction with huntingtin was not affected by the length of the polyQ repeats. In a separate study, HIP-2 was found to interact with 4 RING finger proteins; RNF2, RNF24, HIP1-8 and HSD-4 [164]. Although RNF2 demonstrated E3 activity, it is not known whether huntingtin is an RNF2 substrate. It was not established whether the other RING finger proteins are E3s. Since huntingtin is ubiquitylated, and the known huntingtin interactant HIP-2 can bind 4 separate RING finger proteins, these latter molecules may promote the ubiquitylation and degradation of huntingtin. Confirming this hypothesis will necessitate further detailed investigation.

Spinocerebellar Ataxia Type 1

Spinocerebellar ataxia type 1 (SCA1) is clinically characterised by ataxia, progressive motor dysfunction and loss of brainstem neurons and cerebellar Purkinje cells. PolyQ expansions of the *SCA1* gene, ataxin-1, were found to be responsible for the disorder [165]. Affected neurons of SCA1 patients and transgenic mice contain ubiquitin-positive insoluble nuclear inclusions [166]. Cummings et al. [167] demonstrated that *ataxin-1* is a target for the UPS. Furthermore, although expanded ataxin-1 [92Q] was efficiently polyubiquitylated, it was three times more resistant to degradation than ataxin-1 [2Q]. As part of a transgenic study, this group went on to generate transgenic mice with both an expanded ataxin-1 [82Q] and knock-out of the maternal allele of the gene encoding HECT E3, E6-AP1. These double-mutant mice displayed a more severe pathological phenotype. Neuronal damage occurred at more than four times the rate of the ataxin-1 [82Q] mice despite having less nuclear inclusions than their ataxin-1 [82Q] littermates [167]. These data indicated that impairment of the proteasome rather than the presence of nuclear inclusions was a prerequisite for neu-

rodegeneration. This study provides further evidence that the presence of inclusions may be a cell's attempt to protect itself rather than represent a deleterious mechanism. Surprisingly, E6-AP1 could not ubiquitylate ataxin-1 in vitro [167]. E6-AP1 may require additional but as yet unidentified in vivo factors to target ataxin-1 degradation. Alternatively, the effects observed in the double-mutant mice may be caused indirectly due to E6-AP1 ubiquitylation of ataxin-1-regulatory proteins.

Spinobulbar Muscular Atrophy

Spinobulbar muscular atrophy (SBMA or Kennedy disease) is caused by polyQ expansions in the androgen receptor (AR). The consequence is motor neuronal cell loss and subsequent progressive muscle weakness [168]. The AR is an androgen-dependent member of the steroid receptor superfamily responsible for transcriptional regulation of androgen-responsive genes [169, 170]. The functions of many nuclear receptors including oestrogen receptor alpha are controlled by the UPS [171, 172]. Ubiquitylated nuclear inclusions have been described in motor neurons of SBMA patients and in transgenic mice overexpressing expanded polyQ repeats [173–175]. In cell culture systems, blocking the generation of mutant AR aggregates was found to be deleterious to cells, suggesting cytoprotective properties of such inclusions [84].

AR interacts with 3 RING finger proteins – SNURF/RNF4, ARA54 and ARNIP [176, 177]. Interestingly, each of these RING finger proteins binds to a different domain of AR. SNURF binds to the central domain. This region is required for binding androgen response elements on DNA. This hormone-dependent interaction allows both AR-dependent transactivation and also basal transcription from steroid-regulated promoters [176]. ARA54 is a RING-IBR-RING protein that interacts with the C-terminal steroid specificity domain of AR. ARA54 can enhance the transcriptional activity of wild-type and mutant AR [178]. Perhaps most interesting with respect to SBMA, however, is the interaction between AR and ARNIP (AR N-terminal-interacting protein) [177]. ARNIP, as the name suggests, binds to the N-terminal transcription regulation and polyQ-containing domain. ARNIP interacts with polyQ repeats in both the normal (9–36 repeats) and SBMA disease-associated range (40–62 repeats). In mammalian cells, ARNIP was found to colocalise with AR and in response to androgen stimulation, both were located within the nuclei [177]. Unlike SNURF and ARA54, ARNIP does not appear to act as a coactivator or corepressor of transactivation. By contrast, it displays E3 activity in in vitro ubiquitylation assays [177].

A number of other proteins including BRCA1 and E6-AP1 can act as both E3s and coactivators of the steroid receptor superfamily [179, 180]. Given the complex interplay between SNURF, ARA54, ARNIP and AR, their known functions and the fact that expanded polyQ repeats are commonly more resistant to degradation than the wild-type protein, it is now crucial to establish whether there is an E3 role for these RING finger proteins, in particular ARNIP, in AR-mediated degradation.

Dentatorubral-Pallidoluysian Atrophy

Dentatorubral-pallidoluysian atrophy (DRPLA) is a progressive familial disorder caused by a polyQ expansion in the *atrophin-1* gene on chromosome 12p13.31 [181, 182]. Whereas 6–35 CAG repeats is normal, DRPLA patients have 49–88 [182, 183]. Little is known of the function of mammalian atrophin-1; *Drosophila atrophin-1* (*Grunge*) acts as a transcriptional corepressor with multiple roles in fly development including correct segmentation during embryogenesis [184, 185]. Interestingly, human atrophin-1 forms cytoplasmic lesions that are ubiquitylated in DRPLA but not in control brains [186]. Furthermore, atrophin-1 was found to interact with 3 NEDD4-like HECT domain proteins, i.e. AIP2/WWP2, AIP4/ITCH and AIP5/WWP1 [187]. Both normal and pathological polyQ repeats interacted with these HECT E3s [187]. Although it is yet to be determined, it seems highly likely that atrophin-1 may be a target for AIP-2, -4 or -5 mediated proteolytic destruction given the functional nature of HECT domain proteins. Indeed, similar to observations in HD, disease-associated CAG expansions may be more resistant to degradation than repeats within the normal range, resulting in blockage of proteasomal activity at times of stress [188]. If this is the case, given the high degree of selectivity of the UPS, it is perhaps puzzling that 1 protein should require 3 different E3s to promote its efficient degradation. It raises the possibility that atrophin-1 is an E3-associated protein that aids the target selection. RING E3s can cooperate to catalyse ubiquitylation [189–191]. Moreover, AIP-4 can interact with the RING E3, cCBL to regulate EGFR signalling [192]. Therefore, levels of atrophin-1 may be tightly regulated by different HECT proteins in response to different environmental stimuli.

Prion Diseases

Mutations in the prion protein (PrP) gene are associated with a diverse transmissible, heritable and sporadic group of spongiform neurodegenerative disorders including Gerstmann-Straussler disease and Creutzfeldt-Jakob disease. PrP is a widely expressed cell surface glycoprotein of unknown function [193, 194]. PrP^C, the normal form of the protein, is soluble and highly sensitive to proteases. During the disease process, wild-type or mutant PrP^C undergoes conformational changes to form PrP^{Sc}, which is highly prone to aggregate and form insoluble, protease-resistant inclusions within the cytoplasm of neuronal cells [193–195].

Mahogunin is a RING E3 responsible for the *mahoganoid* coat colour mutation in mice [196, 197]. Mice carrying a null mutation for *mahoganoid* developed age-dependent spongiform neuropathology as well as an altered coat colour. This pathology resembled that observed in prion diseases but lacked the accumulation of protease-resistant PrP [197]. PrP itself does not appear to be a target for mahogunin-mediated ubiquitylation and degradation [197]. It will be interesting to see whether disease-associated mutant PrP or regulators of PrP metabolism are mahogunin targets of the UPS.

Lafora Disease

Lafora disease (LD) is the most common and severe form of adolescent-onset progressive myoclonus epilepsy. This autosomal recessive disorder causes rapid and severe neurodegeneration, ataxia and myoclonus epilepsy. Affected individuals usually fail to survive more than 10 years after onset. The disease is characterised by an accumulation of starch-like polyglucosans, or Lafora bodies, within the endoplasmic reticulum in both neuronal and non-neuronal cell types [198–200]. These inclusions are found in dendrites but not axons [200, 201]. Interestingly, they are ubiquitin positive exclusively in neuronal cells, suggesting different processing and pathological consequences for Lafora inclusions in these tissues [198, 199].

To date, 2 genes have been identified with linkage to the disorder [200, 202]. 70% of individuals with LD carry mutations in *EPM2A*, on chromosome 6q24 that encodes laforin, a dual-specificity phosphatase [201, 202]. Generation of *Epm2a* knockout mice demonstrated that neuronal degeneration and Lafora inclusion bodies predate the onset of impaired behavioural responses associated with this disease [199]. Chan et al. [200] have recently

identified a second locus responsible for LD, *EPM2B* (*NHLRC1*), which encodes malin, a RING-finger-containing protein. Although little is known about the role of malin, given the presence of the RING motif, it may function as an E3. Disease-associated mutations in malin may lead to the accumulation of polyglucosans and dendritic protein targets causing disruption of neuronal synaptic function and subsequent dementia, neurologic deterioration and epilepsy.

Other Components of the UPS Associated with Neurodegenerative Disorders

Several other UPS proteins have been identified that may be linked to the development of a number of other neurodegenerative conditions. Machado-Joseph disease is a polyQ disease caused by expansions of the ataxin-3 protein. Ataxin-3 can interact with DNA repair proteins HHR23A and HHR23B via their ubiquitin-like domains [203] and possesses DUB activity [204]. In addition, ataxin-7, a polyQ-containing protein associated with spinocerebellar ataxia type 7, associates with the 19S proteasomal subunit S4 [205].

Ubiquitin-related proteins such as SUMO, and the ubiquitin-binding protein p62, are commonly found in the pathological inclusions associated with neurodegenerative diseases [206–209]. In AD, A β may be involved in up-regulating ubiquitylation and/or down-regulating proteasome activity [210–212]. Mutant α -synuclein, associated with familial PD, is also thought to down-regulate proteasome activity [9, 141, 143]. Furthermore, proteolysis of both tau and α -synuclein can occur via proteasomal mechanisms independently of ubiquitylation [213, 214].

Another layer of complexity associated with AD, and possibly other neurodegenerative disorders, is molecular misreading [215–219]. This age-related process occurs through the generation of a transcript from a normal gene, but which loses one or more base pairs during the transcriptional process. In AD patients, novel frameshift mutations in the *ubiquitin B* gene lead to the translation of novel but nonsense peptide sequences creating ‘ubiquitin +1’. Although ubiquitin +1 can act as an acceptor for additional ubiquitin molecules, it cannot donate itself to an expanding polyubiquitin chain as it has lost its C-terminal glycine residue. This mutant form of ubiquitin blocks proteasomal degradation [219] and causes neuronal cell death [217].

Concluding Remarks

Ubiquitin-proteasome-mediated processes clearly play an important role in many neurological disorders. Recent elucidation of RING motif-containing proteins, the fourth most abundant protein domain in the human genome, as a major class of E3s has rapidly broadened our understanding of many disease processes over the past few years. Neurodegenerative diseases are no exception to this and a number of E3 proteins have been identified to be associated with neurodegeneration. Although enzymatic activity of a number of others need to be confirmed, these proteins are highly likely to be E3s. Obviously, it is now paramount that these proposals are confirmed *in vivo*.

The molecular basis for the tissue-specific effects observed as a result of a malfunctioning UPS are not understood. Many E3s are ubiquitously expressed and they can target different substrates in the same cell. Tissue-specific effects may be exacerbated in neurons as they are terminally differentiated cells and generally do not self-renew. Moreover, there may be degeneracy in substrate targeting in different cells by different E3s. However, these possibilities cannot fully explain why specific populations of neuronal cells are targeted for damage and/or loss in a given neurodegenerative disorder. For example, PD is associated with loss of the dopaminergic cells of the substantia nigra in both sporadic and familial forms of disease including AR-JP. Parkin, mutations in which cause AR-JP, is found in multiple tissues, yet mutant parkin only exerts obvious phenotypic effects in the substantia nigra.

Why? Studies aimed at identifying which key substrate, of potentially many, of a particular E3 is not processed appropriately will be of paramount importance in answering such questions.

From a treatment perspective, it is exciting to realise that inappropriate activity of a single E3 may be associated with more than one disease. For example, dorfín can ubiquitylate proteins associated with both PD and ALS; parkin plays a crucial role in PD, but may also be active in polyQ disorders. Furthermore, more than one E3 may be associated with a particular disease. From our current understanding, parkin appears to be the principal E3 associated with hereditary PD. What are the roles, if any, of dorfín, Siah-1 or other E3s in disease progression? CHIP can enhance parkin E3 activity. Perhaps other E3s can also interact to enhance/inhibit specific ubiquitylation events during PD.

Understanding the complex mechanisms involved in the recognition and specific targeting of both normal and mutant proteins may provide insights into how we can develop therapeutic strategies to degrade precisely a single protein within a diseased cell [21].

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