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THE BIOCHEMISTRY OF PARKINSON'S DISEASE*

Mark R. Cookson
Cell Biology Section, Laboratory of Neurogenetics, National Institute on Aging, Bethesda, Maryland 20892; email: cookson@mail.nih.gov

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■ **Abstract** Several genes have been identified for monogenic disorders that variably resemble Parkinson's disease. Dominant mutations in the gene encoding α-synuclein enhance the propensity of this protein to aggregate. As a consequence, these patients have a widespread disease with protein inclusion bodies in several brain areas. In contrast, mutations in several recessive genes (*parkin*, *DJ-1*, and *PINK1*) produce neuronal cell loss but generally without protein aggregation pathology. Progress has been made in understanding some of the mechanisms of toxicity: Parkin is an E3 ubiquitin ligase and DJ-1 and PINK1 appear to protect against mitochondrial damage. However, we have not yet fully resolved how the recessive genes relate to α-synuclein, or whether they represent different ways to induce a similar phenotype.

CONTENTS

DEFINITIONS 29
α-SYNUCLEIN AND AGGREGATION 31
PARKIN AND THE PROTEASOME 38
DJ-1 AND PINK1: MITOCHONDRIAL CONNECTIONS? 42
PATHWAYS TO PARKINSONISM 44
CONCLUDING REMARKS 47

DEFINITIONS

For many of us, our opinions about diseases are greatly informed by the infectious diseases, where we can rely on Koch's postulates to tell us causation. We can isolate and culture the microorganism responsible for infection, introduce it into a host, and see the disease recapitulated. However, these ideas are less useful in understanding pathogenesis in the neurodegenerative disorders in which sporadic cases occur without readily identifiable causes. We can circumvent this to some extent by examining monogenic forms of the disease. Here there is a much simpler

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cause and effect relationship: inherit a faulty copy (or copies) of the gene and develop disease. Knowing the gene identity and the nature of mutations, one can introduce these into animals or other models and induce a facsimile of the disease. An underlying assumption is that the disease process might also be similar for the sporadic forms of these disorders.

There is a special problem of definition when discussing Parkinson's disease (PD). Although there are multiple loci with PARK designation (Table 1), the

TABLE 1 Monogenic forms of Parkinson's disease

Locus[a]	Gene	Protein function	Phenotype[b]
PARK1	*SNCA*[c]	Synaptic?	PD/DLBD
PARK4 (AD)		Lipid binding?	Onset from age 30 to 60, rapid course. Fulminant Lewy bodies
PARK2 (AR)	*Parkin*	E3 ligase	Parkinsonism Onset from teenage to 40s, slow course. No Lewy bodies, except one case
PARK3 (AD)	Unknown; Chr2p13[d]	—	PD, dementia Onset from age 50 to 60s Lewy bodies, tangles and plaques.
PARK5 (AD?)	*UCHL1*	Ubiquitin hydrolase/ligase	Typical PD Onset at about age 50 Unknown pathology
PARK6 (AR)	*PINK1*	Protein kinase	Parkinsonism Onset from age 30 to 50s Unknown pathology
PARK7 (AR)	*DJ-1*	Oxidative stress response?	Parkinsonism Onset from age 20 to 40s, slow course. Unknown pathology
PARK8 (AD)	Unknown; Chr12p1-q13	—	Parkinsonism Onset from age 40 to 60s Variable pathology[e]

[a]PARK loci are shown for the monogenic forms. Inheritance is shown in parentheses below each loci: AD, autosomal dominant; and AR, autosomal recessive.

[b]PD, Parkinson's disease; DLBD, diffuse Lewy body disease.

[c]*SNCA* is the gene name for α-synuclein.

[d]The PARK3 linkage was described in Reference 148.

[e]PARK8-linked families have been described by two groups as either Lewy body negative or variably Lewy body and tangle positive. See References 149–150.

syndromes for each are distinct. The genes for six of the loci are known and will be discussed in the text. Additionally, there are two extant loci for which the underlying genetic lesion has not been identified. Each gene will be discussed separately, but broadly there are two clinicopathological components. Firstly, there is clinically defined parkinsonism, a syndromic term which encompasses the cardinal features of the Parkinsonian movement disorder. These are a resting tremor, bradykinesia (slowness of movement), rigidity and postural instability, all problems in initiating or stopping movement. Their pathological correlate is the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Although there are many other neuronal groups affected in different brain regions in PD (1), nigral degeneration and resultant parkinsonism is a consistent feature of all the genetic conditions discussed here. Secondly, PD is marked postmortem by the presence of Lewy bodies (LBs) and Lewy neurites in surviving neurons (2). These are intracellular aggregations of lipids and proteins that were first identified by eosin staining and now by immunostaining for their protein components including ubiquitin and α-synuclein. Pathological definitions of PD require the presence of α-synuclein-positive Lewy pathology in surviving nigral neurons, combined with nigral cell loss and intact striatal neurons (3). α-Synuclein also marks the lesions found in a range of related disorders, referred to as synucleinopathies (4). Importantly, in diffuse Lewy body disease (DLBD), Lewy pathology is found in many brain areas and is associated with the expanded phenotype of these cases, such as dementing illness and fluctuations in consciousness (5).

PD is therefore a disease with two parts, parkinsonism and Lewy pathology. Neither of these alone precisely defines PD as both occur independently in other neurological settings. However, understanding these two components is critical to understanding the biochemistry of PD and how the genetic forms of parkinsonism relate to this process. Conveniently, α-synuclein is both part of Lewy pathology and a cause of dominantly inherited disease.

α-SYNUCLEIN AND AGGREGATION

In 1997, Polymeropolous and colleagues identified a dominant mutation in the α-synuclein gene in a number of families of Greek or Sicilian ancestry (6). This gene had been cloned previously as a precursor to a small peptide found in the Alzheimer's brain (7). Other homologues had been cloned as a gene upregulated by song learning in the zebra finch (8) or as an abundant protein from the electric eel neuromuscular synapse (9), where the protein was named for its localization to synapses and nuclei. In fact, α-synuclein is part of a gene family including β- and γ-synucleins and synoretin (10). All synucleins have a series of imperfect repeats including the sequence motif KTKEGV and a variable C-terminal tail, which is highly acidic in α-synuclein. Synucleins are also basally phosphorylated at serine and tyrosine residues. Some of these structural motifs in α-synuclein are shown in Figure 1. There is little or no detectable secondary structure in solution, and hence α-synuclein is referred to as natively unfolded.

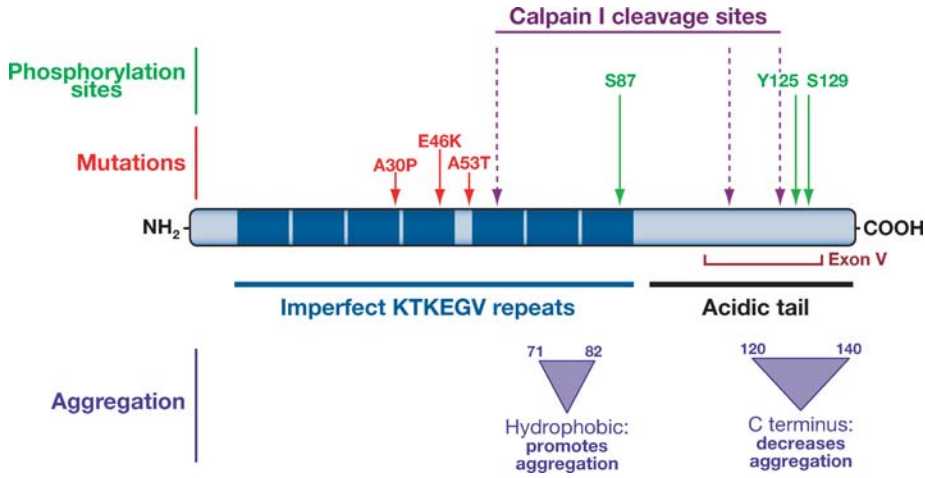


Figure 1 Motifs in the α-synuclein protein. The natively unfolded α-synuclein protein is shown in a linear form. PINK1 shaded areas represent the imperfect KTKEGV repeats. Human mutations are shown in red and map to the repeat region. At the C-terminal end of the protein is an acidic tail, containing several sites of phosphorylation (*green*). The C terminus also contains the alternatively spliced exon V and a calpain I cleavage site. The acidic tail tends to decrease protein aggregation, whereas a hydrophobic region near the imperfect repeats promotes aggregation.

The functions of the synucleins are not well understood. α-Synuclein binds to lipid membranes, forming an amphipathic helix (11). Given the location of a pool of α-synuclein at synaptic membranes, there may be a synaptic role for the protein. In support of this idea, α-synuclein knockout mice have synaptic deficits. An early report suggested a loss of dopamine release in the striatum (12), whereas a more recent study showed a decrease in paired-pulse facilitation in the hippocampus (13). This may correlate with a loss of synaptic markers in antisense-treated hippocampal cultures (14). Taken together, these observations suggest that α-synuclein plays a role in regulating the reserve pool of synaptic vesicles in brain. Possibly related to lipid binding, α- and β-synucleins also inhibit phospholipase D2 at physiological concentrations (15, 16). However, knockout animals show no PD-like symptoms, suggesting that loss of protein function does not cause disease.

The first α-synuclein mutation that was discovered is an A53T point substitution (6). An unusual aspect of the mutation is that the amino acid is already a threonine in rodents and other species (17). Subsequently, three additional mutations have been found: A30P in German kindred (18), E46K in a Spanish kindred (19), and a triplication of the wild-type gene in a large family from Iowa (20). Pathology from three of these kindreds is available and shows α-synuclein-positive Lewy bodies in the brainstem as well as nigral cell loss. However, α-synuclein pathology is not limited to the nigra in many of these cases. In fact, the clinical descriptions of

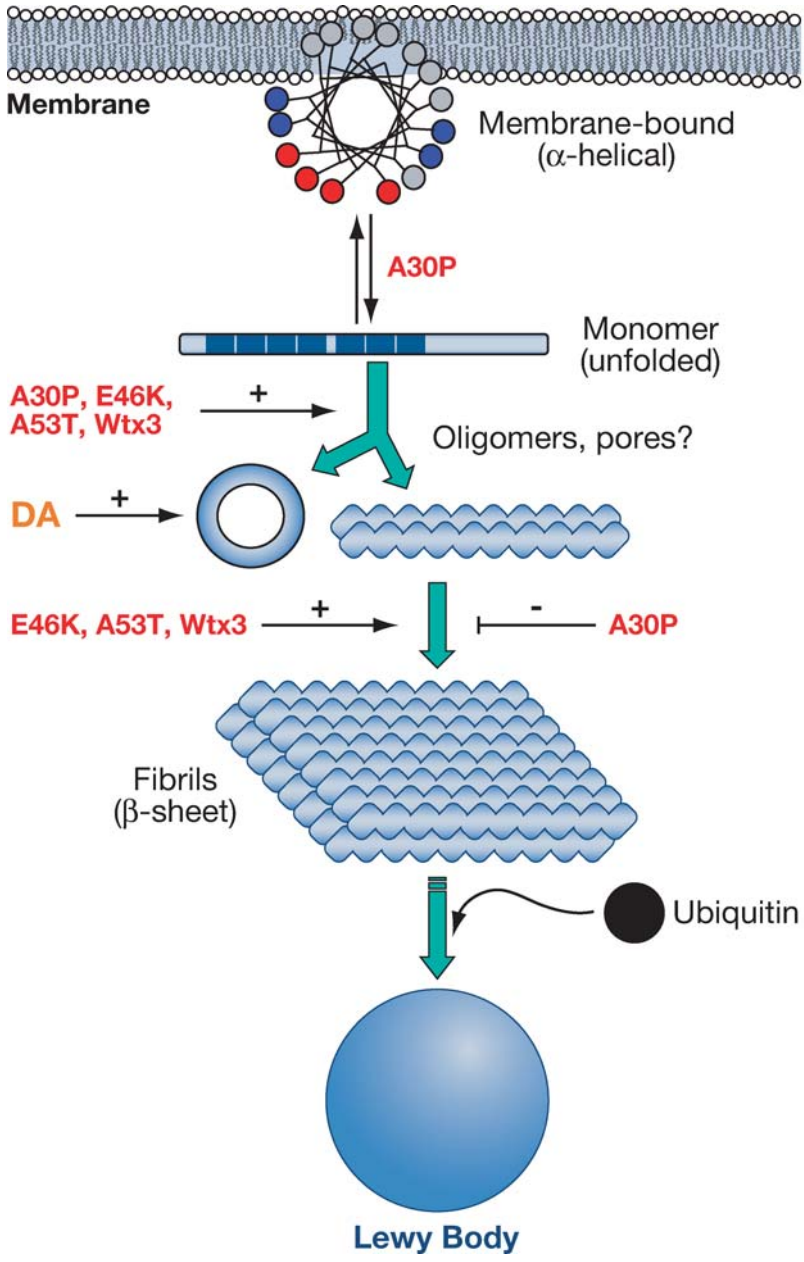
many of the patients with α-synuclein mutations reflect a disease with prominent dementia, presumably a reflection of the widespread cortical Lewy bodies in these cases. The Spanish E46K mutation was reported as "Lewy body dementia" (19). In the Iowan kindred, glial cell inclusions were found (21), which would otherwise be typical of multiple system atrophy (MSA), another synucleinopathy. Therefore, mutations in α-synuclein produce a fulminant disease that includes parkinsonism but is much more widespread and may resemble DLBD. The disorder is also more progressive and tends to have an earlier onset than sporadic PD.

α-Synuclein is one of several proteins associated with neurodegenerative diseases that have a high propensity to aggregate. β-pleated sheet-like bonding stabilizes the aggregated forms. This contrasts with the unstructured protein in solution or folding when bound to lipid, earning α-synuclein the title of "a protein chameleon" (22). A central hydrophobic region of α-synuclein, near the repeats, tends to self-associate, contributing to aggregation (23, 24). This region is not shared with (for example) β-synuclein, and consequently these homologues vary radically in their propensity to aggregate. In fact, β-synuclein can prevent α-synuclein aggregation in vivo (25) and in vitro (26). The C-terminal acidic tail of α-synuclein inhibits aggregation and, hence, truncated forms are more prone to aggregate (27, 28). This ability of wild-type α-synuclein to aggregate presumably explains its presence in the several sporadic synucleinopathies.

The end product of α-synuclein aggregation is the formation of heavily insoluble polymers of protein known as fibrils. It is thought that fibrillar α-synuclein is the building block of Lewy bodies. The most direct evidence for this is immunogold labeling showing that α-synuclein is present at sites along fibrils isolated from Lewy bodies (29). Lewy bodies contain many proteins other than α-synuclein, including neurofilaments and other cytoskeletal proteins, suggesting there are co-precipitants that might be important in aggregation. However, fibrils can be formed in vitro from α-synuclein alone, suggesting that this protein is sufficient to form inclusions [reviewed in (22)]. α-Synuclein is also the most sensitive marker for Lewy bodies, implying that it is necessary for Lewy body formation (30).

Although the A53T mutation promotes the formation of such fibrillar species, A30P does not. In fact, A30P slows the rate of fibril accumulation but strongly promotes the formation of oligomeric species (31, 32). No studies on the E46K mutation have been performed to date, but the pathology in these cases suggests the mutation would have an effect on fibril formation. Because aggregation is a concentration- and nucleation-dependent process (33), the Iowan triplication is predicted to promote accumulation of oligomers and fibrils. We have recently shown that oligomeric α-synuclein is deposited in detergent-insoluble fractions from brains of patients with the triplication mutation (34). Figure 2 shows an outline of the α-synuclein aggregation pathway, with an emphasis on the effects of different mutations.

The fact that all mutations promote the formation of oligomeric rather than fibrillar species has led some to suggest that oligomers, not fibrils, are toxic. Oligomers, also referred to as protofibrils (35), can form annular structures that



may have pore-like properties and might damage membranes (36). Recently, annular synuclein oligomers have been isolated from human brain samples (37). There is some additional evidence to support the idea that oligomers are the toxic species for α-synuclein. In most cell culture models, toxicity is seen without heavily aggregated α-synuclein, and it has been suggested that soluble species mediate toxicity (38). α-Synuclein aggregation and deposition into insoluble fractions occurs later than cell death in vitro (39). Conversely, Lewy body formation involves deposits of fibrillar α-synuclein into very insoluble fractions. Therefore, α-synuclein aggregation is the key step that drives both pathology and cellular damage, but these two outcomes can be dissociated from each other.

Common effects between mutations, such as the formation of oligomers, are important to note, as they are more likely to be related to the pathogenic mechanism than differential effects. However, this is true only if both mutations are unambiguously causal. The A30P family is relatively small, leading to concerns about whether the mutation is truly causal, although the mutation does segregate with disease with a reasonably high penetrance (40). A second difficulty is the lack of autopsy studies. Positron emission tomography data show presynaptic nigral cell loss, and the clinical phenotype is reported to be "to that of sporadic" PD (40). However, as PD is defined primarily by pathology, the diagnostic accuracy is unclear. If oligomeric species mediate toxicity to neurons and A30P promotes only oligomer formation, and if Lewy bodies are a consequence of the formation of fibrillar species, then one might predict that the A30P family would have much fewer Lewy bodies than the other mutations and might be defined as parkinsonism not PD. However, assuming that the A30P mutation is causal, then all of the α-synuclein mutations share the same causal agent and so have the same etiology.

It is clear from the above discussion that protein aggregation is important in the disease, and hence it is critical to elucidate both genetic and nongenetic factors that increase this aggregation. Promoter alleles that increase expression of

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Figure 2 The pathogenic cascade of α-synuclein aggregation. α-Synuclein exists in solution as an unstructured monomer, shown as a linear structure, similar to Figure 1. Inside the cell, the monomer is in equilibrium with membrane-associated forms with higher helical content, shown schematically as an amphipathic helix. In the helix, blue and red circles indicate charged residues, gray circles are nonpolar and hydrophobic amino acids. The A30P mutation disfavors membrane binding. The green arrows indicate the pathogenic formation of aggregated species. All mutations reported to date increase the rate of formation of oligomers or protofibrils, which may also produce pores. Oligomers and other intermediates are kinetically stabilized by dopamine (DA). However, these are transient species that further aggregate to form mature fibrils, which are stabilized by β-sheet-like interactions and are highly insoluble. The formation of Lewy bodies is presumed to be a consequence of fibrillization. Events such as the attachment of ubiquitin (*black dot* in the figure) are thought to be secondary to the initial aggregation and deposition processes.

α-synuclein (41), therefore increasing protein concentration, are associated with sporadic PD (42). Metals, pesticides, and oxidizing conditions all promote α-synuclein aggregation [reviewed in (43)], suggesting a reason why these appear to play a role in sporadic PD. α-Synuclein also undergoes a number of posttranslational modifications, some of which enhance aggregation. The phosphorylated form of α-synuclein is found in Lewy bodies (44). However, given that α-synuclein is basally phosphorylated, whether phosphorylation precedes aggregation and/or deposition is unclear. Tyrosine nitration (45) and methionine oxidation (46, 47) also occur, although again whether nitration or oxidation is required for α-synuclein toxicity has not been directly tested. Finally, cleavage of α-synuclein by calpain I is also associated with deposition (48). Because the C-terminal region of the protein prevents aggregation, removal is predicted to promote aggregation. Calpain I cleavage has also been associated with neuroprotection in vitro (49).

Although α-synuclein is expressed in many tissues, symptoms are restricted to particular neuronal cells. Part of the reason is that aggregation does not occur equally in all tissues. For example, α-synuclein is not as heavily aggregated in blood samples as in the brain from patients with the triplication mutation (34). Why is the brain vulnerable to these processes when other tissues also express the same protein? There are likely to be several contributing factors. Expression of α-synuclein is high in the brain compared to other tissue (50). Brain also has a high concentration of macromolecules, which may promote aggregation by molecular crowding (51, 52). The brain may have a higher oxidative stress level than other tissues. Some neuronal groups may be highly vulnerable because catechols, including dopamine, can stabilize oligomeric intermediates of aggregation (53). Conversely, inhibition of dopamine synthesis ameliorates α-synuclein toxicity (38). These observations reinforce the idea that oligomeric α-synuclein species are toxic.

One aspect that deserves special discussion is the potential role of lipids. As stated above, α-synuclein adopts a helical conformation when bound to lipid membranes. This is a fairly stable conformation, which would inhibit conversion of protein into fibrillar forms (54). However, lipids can also promote α-synuclein aggregation, especially forming oligomeric species (55, 56). If oligomers were the toxic species, then lipids would promote α-synuclein toxicity. In support of this, genes that suppress α-synuclein toxicity in yeast are clustered in families related to lipid metabolism and vesicle transport (57). Relating this to human pathology, Lewy bodies contain lipids at their core (58). The A30P mutation, but not A53T, decreases α-synuclein's affinity for artificial lipid membranes (59) and promotes its cytosolic accumulation in yeast (57).

This discussion suggests that α-synuclein is the causative agent in PD/DLBD. Stretching the analogy to Koch's postulates further, can we reintroduce α-synuclein mutations into a susceptible organism and reproduce the disease? α-Synuclein transgenic animals have been made in a number of species, including mice, *Drosophila melanogaster*, and *Caenorhabditis elegans* [reviewed in (1, 60)]. Viral delivery of α-synuclein induces nigral degeneration in rats (61) and primates (62).

These apparently solve the problem of whether α-synuclein is the causal agent, as the disease is replicated in the model organism.

A difficulty is that the point mutations apparently behave differently in different contexts. For example, in two models, A53T α-synuclein promotes damage to the spinal cord and leads to complex motor phenotypes without nigral cell loss (63, 64). Despite matched levels of expression, A30P α-synuclein does not produce an equivalent phenotype. This observation has been used to suggest either that A30P is not pathogenic (64) or that fibrils are more toxic than protofibrils (65). However, in other models A30P produces several effects, including intracellular inclusions and neuronal damage (66). In *Drosophila*, A30P is more toxic than wild type or A53T (67). Further complicating the picture, nigral neurons remain intact in all of the transgenic mouse models to date, but loss of dopaminergic neurons is seen in transient, viral-induced experiments in rats or primates. Why these models are quite so variable is unclear.

An important point in thinking about the different mutations in α-synuclein is that the difference between wild-type and mutant proteins is quantitative rather than qualitative. In other words, the properties of α-synuclein that cause damage to neurons are not specific to the mutants but are already a function of the wild-type protein. This is neatly illustrated in experiments using inducible promoters to drive α-synuclein expression and toxicity in human cell lines (38). At lower expression levels, mutant α-synuclein is more toxic than wild type, but at higher expression levels, all variants are similarly damaging. Although it is difficult to directly compare the different transgenic models, mutant proteins are associated with greater amounts of pathology until the wild-type protein is expressed at high levels. The corresponding dosage curve for human studies may come from studies of multiplication mutations. The triplication, with double the protein load, includes Lewy pathology and cellular damage in many brain regions. At the other end of the range, different promoter variants may more subtly affect expression (42) and are associated with idiopathic PD.

In summary, α-synuclein is a protein with a natural tendency to aggregate into oligomers that can then further aggregate into fibrils that are deposited as Lewy bodies and similar pathologies. Mutations variably produce either oligomers/protofibrils or fibrils. The fibrillar mutations (A53T, triplication of the wild-type alleles, and probably E46K) produce a widespread Lewy body disease in humans that includes parkinsonism but also has elements of DLBD. PD/DLBD is therefore a protein aggregation disorder, which could lead to therapeutics aimed at preventing or reversing α-synuclein aggregation. For example, peptides directed at the central portion of α-synuclein can prevent or limit aggregation and toxicity (68), and antibodies to aggregated α-synuclein (69) may also be beneficial if they can be expressed inside cells. There are other possible routes to interfere in the pathogenic process, such as any of the downstream effects caused by the presence of aggregated proteins in a neuron. Because these might overlap with the cellular functions impacted by the recessive mutations associated with inherited parkinsonism, these are discussed in the next two sections.

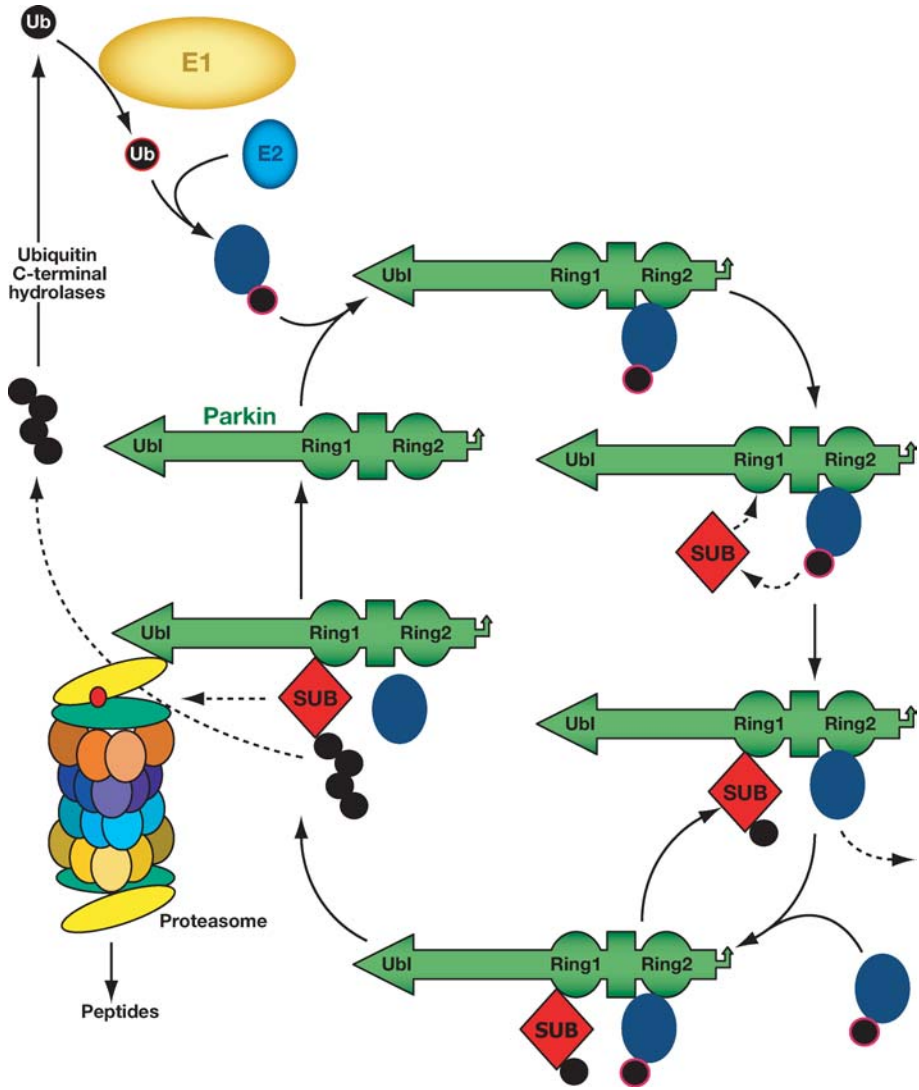
PARKIN AND THE PROTEASOME

The first gene that causes recessive parkinsonism was named *parkin* by Mizuno and colleagues (70). These patients had early-onset parkinsonism (teens to twenties) with slow progression and additional features such as dystonia. Subsequent studies suggest that *parkin* mutations are the most numerous cause of recessive, early-onset parkinsonism [reviewed in (71)]. Identification of additional mutations shows that the phenotype can be expanded to include cases with features more typical of sporadic PD (72). One discrepancy between *parkin* disease and PD is the absence of Lewy bodies, although there is one exception (73). This suggests Lewy body formation is not required to evoke nigral cell death, i.e., that there are other ways to kill neurons. So *parkin* disease is a phenocopy of PD, parkinsonism without Lewy bodies. There are other forms of parkinsonism with these features, notably exposure to the toxin 1-methyl-4-phenyl-1,2,4,6,-tetrahydropyridinium (MPTP) (74).

Subsequent studies have shown that *parkin* encodes an E3 protein-ubiquitin ligase (75, 76). E3 ligases control the key step in the cycle of ubiquitin-mediated hydrolysis of damaged or misfolded proteins that are degraded via the proteasome. The reaction promoted by E3 ligases is the addition of a lysine-linked chain of four or more ubiquitin molecules to the target protein, which is recognized by subunits in the proteasome lid. An outline of the reaction scheme catalyzed by parkin is shown in Figure 3. Parkin's domain structure reflects this role. At the N terminus of parkin is a ubiquitin-like domain (Ubl) that interacts with Rpn10

Figure 3 A parkin-centric view of the ubiquitin-proteasome system. Ubiquitin is activated by the enzyme E1 (*yellow*), represented here by a red circle around the black dot of ubiquitin. After activation, ubiquitin is transferred to an E2 enzyme (*blue*), which docks with E3s including parkin (*green*). Parkin contains two RING (really interesting new gene) domains separated by an IBR (in-between ring) motif, and the E2 is probably recruited to this region. Substrates (*red diamond*; see text for description of the different candidates) then bind to the same region of parkin. For simplicity, substrates are shown binding to RING1 and E2 to RING2, but as there are no data on how parkin is folded, we cannot be sure of the exact spatial arrangement of these components. Activated ubiquitin is transferred from the E2 to the target and, by analogy to other E3 ligases, there is no transfer to parkin itself. This process is repeated until a string of four or more ubiquitin molecules, linked by lysines to each other and to the substrate, is formed. This is recognized by the proteasome, which degrades the protein into small peptides and amino acids. During all or some of this process, Parkin may be tethered to the proteasome by interactions of its N-terminal ubiquitin-like (Ubl) domain. Prior to substrate degradation, the polyubiquitin chain is removed and recycled to monomeric ubiquitin by a series of enzymes including the ubiquitin C-terminal hydrolases. Parkin is also freed to participate in further reactions.

in the proteasome cap (77). It is likely that the Ubl domain tethers parkin close to the proteasome, directing poly-ubiquitylated proteins toward their proteolytic end. At least one recessive parkin mutation (R42P) disrupts this interaction (77). At the C-terminal region of parkin, there are two RING (Really Interesting New Gene) fingers. RING fingers are found in a number of E3 ligases and have varying numbers of cysteine and histidine residues that coordinate a structurally important zinc atom (78). Parkin has two RING fingers of the C_3HC_4 type separated by an in-between RING (IBR) domain, again typical of E3 ligases. The RING-IBR-RING



motif is important in recruiting substrates and the E2 enzyme (UbcH7 or UbcH8) that carries activated ubiquitin. The precise mechanism by which parkin promotes ubiquitylation of its substrates is not fully defined, in part because the structure of parkin has not been solved. However, other E3 ligases that have similar RING domains act as scaffolds to bring the ubiquitin bound to the E2 close to target lysines on the substrate protein. These E3 ligases are not catalysts as the reaction does not proceed via a thiol intermediate as on the E3 but is based on a proximity effect. A by-product of this reaction scheme is that these E3 enzymes undergo autoubiquitylation, a phenomenon that is easily seen with parkin.

There are several targets for parkin's E3 ligase activity. These include the septins CDC-rel1 and CDC-rel2 (75, 79), cyclinE (80), p38 tRNA synthase (81), Paelr1 (82), synaptotagmin XI (83), and synphilin-1 (84), as well as parkin itself (75, 76). It is notable that some of these proteins are synaptic, as parkin is tethered to synaptic densities by an interaction with the PDZ protein Cask (85). This implies that parkin could have a role in synaptic function. In most cases, parkin can ubiquitylate substrates without additional binding proteins, but parkin does require an additional protein (hSel10) for activity against cyclin E (80). Parkin may be present as part of a modular complex in the brain with additional proteins that act to control substrate specificity. At least one additional protein can also bind to parkin, the U-box protein CHIP (86). CHIP interacts with Hsp70 to ubiquitylate misfolded proteins. Hsp70 and CHIP are important components of the decision-making machinery of the cell to direct either protein refolding in the endoplasmic reticulum (ER) or retrotranslocation to the cytosol and degradation.

There are suggestions that parkin inactivation may play a role in typical PD. Two groups have recently shown that exposure to nitric oxide (NO) alters parkin's E3 ligase activity (87, 88). The mechanism involves NO-derived radical species that attack cysteine residues in RING1. As these cysteines are important in coordinating the structural zinc molecule, this will affect protein folding and, hence, enzyme activity. In one study, decreased activity of parkin toward synphilin-1 was noted (87), whereas increased autoubiquitylation was seen in the other study (88). Whether small differences in experimental conditions are responsible for these apparent discrepancies remains to be resolved. One notable difference is that shorter exposures to nitrosylating agents produce increased activity, whereas longer exposures inhibit activity. Parkin is nitrosylated both in human tissue from PD patients and in the MPTP and rotenone animal models (87, 88). NO-derived radicals are important mediators of MPTP toxicity (1). Another observation suggesting parkin may play a role in sporadic PD is the reported association with promoter polymorphisms (89), although conflicting results have been reported (90).

By implication, loss of parkin function results in the accumulation of one or more of its substrates, which then leads to cell death of nigral neurons. Support for this idea comes from experiments where overexpression of the parkin substrates Paelr1 (91) produces dopaminergic cell death that can be rescued by parkin but not its E3 inactive mutants. Another substrate, CDC-rel1, also causes cell death restricted to the nigra in vivo (92). One genetic finding that has been widely

discussed in support of the proteasome hypothesis is the mutations in *UCHL1* in two siblings with PD (93). This gene encodes a neuronal-specific ubiquitin C-terminal hydrolase, whose enzymatic roles include the recycling of ubiquitin chains back to monomeric ubiquitin and adding ubiquitin to already monoubiquitylated α-synuclein (94). UCHL1 expression is restricted to neurons, making it an interesting candidate for neurodegeneration. However, there is controversy with the *UCHL1* mutation, discussed in an excellent recent review (95). There have been no additional families with UCHL1 mutations, despite extensive searches, that would strengthen the case that this gene can be pathogenic for PD. Therefore, whether *UCHL1* mutations are truly causal for PD is unclear. There is, however, a relatively common S18Y polymorphism in *UCHL1* that has been associated with risk for sporadic PD in several, but not all, studies (95). It is possible that UCHL1 plays some role in PD, but this requires further clarification.

Parkin is also neuroprotective against stresses in which the direct relationship to its substrates is unclear. Because of its unambiguous contribution to dominantly inherited PD, several laboratories have examined whether there is a relationship between α-synuclein and parkin. For example, we showed that parkin, but not its E3 inactive mutants, protects cells against mutant α-synuclein (96), which has been confirmed in other laboratories (49, 87). Others have shown that parkin suppresses mutant α-synuclein toxicity in *Drosophila* models (91, 97). Demonstrating again that the difference between wild-type and mutant α-synuclein is qualitative rather than quantitative, parkin can suppress the toxicity associated with expression of high levels of α-synuclein in vitro (98).

The simplest explanation for this observation is that α-synuclein might be a parkin substrate. The steady-state level of α-synuclein protein is not affected by the expression of parkin in cell lines (84) or in *Drosophila* (91). Although there have been suggestions that α-synuclein protein levels might respond to proteasome inhibition in vitro, most studies have not noted any effect. One study suggested that α-synuclein can be degraded by the proteasome in an ubiquitin-independent fashion (99), which would not require an E3. There is also evidence for α-synuclein degradation by lysosomal proteases (100, 101). Set against these observations is the experimental evidence that there is a glycosylated form of α-synuclein, sp22, that can be purified from human brain and is a substrate for parkin (102). To date, there are no replications of this important result, so it is not quite clear whether sp22 is present in some of the model systems where α-synuclein is demonstrably toxic and, hence, whether formation of sp22 is required for α-synuclein toxicity. If the relationship between parkin and α-synuclein is not one of enzyme and substrate, then what are the mechanisms involved in neuroprotection? There are several possibilities, which will be discussed later in this chapter.

Creating knockout models for parkin is one way to model the disease and understand the pathogenic process. Two groups have produced mice with targeted deletion of exon 3 of *parkin*. Neither shows loss of nigral neurons, although there are subtle changes in dopaminergic neurotransmission (103, 104). Recently, mice with an exon 7 deletion have been reported to have loss of neurons in the

locus coeruleus with attendant behavioral changes (105). Although there is no loss of catecholaminergic neurons, *Drosophila* parkin knockouts show mitochondrial damage and apoptosis of flight muscles (106). Interestingly, one of the knockout mouse models shows deficits in mitochondrial respiration (107). Perhaps surprisingly, then, the major phenotype reported in parkin models is mitochondrial. Parkin rather specifically prevents mitochondrial cytochrome *c* release and apoptosis in vitro (108). This observation has gained greater weight as it has been realized that other recessive genes for parkinsonism also impact on mitochondrial function.

DJ-1 AND PINK1: MITOCHONDRIAL CONNECTIONS?

Since the discovery of parkin mutations, two additional genes have been found associated with recessive parkinsonism, DJ-1 (109) and PINK1 (110). Patients with mutations in either of these two genes have similar phenotypes to each other and to parkin. Onset is generally early (from ages 30 to 50), and the course is benign with long disease duration. Individuals with DJ-1 (111) or PINK1 mutations (112) have loss of presynaptic dopaminergic function, although no autopsy studies are yet available.

Because it is recessive, we might expect mutations in DJ-1 to be loss-of-function mutations. Hence, understanding this normal function is critical. DJ-1 was cloned as an interactor of GAPDH, playing a role in mRNA regulation by stabilizing mRNA species after transcription (113). DJ-1 becomes more acidic under oxidative conditions, one of only several proteins that do so (114). These two facts led Bonifati and colleagues (109) to suggest that DJ-1 maintians neuronal viability by modulating gene expression under conditions of cellular stress. DJ-1 has limited homology to several prokaryotic proteins, including cysteine proteases and chaperones (115). Weak protease (116) and chaperone (117) activities have been reported, although whether these are physiologically relevant has not been established. Several groups have crystallized the DJ-1 dimer, and the structure indicates that while there is a cysteine (C106) that might be catalytically active (117), it is not adjacent to a histidine residue as seen in authentic proteases in the superfamily (118).

The first description of DJ-1 mutations suggested that one point mutation, L166P, promotes localization of the normally cytoplasmic DJ-1 protein to mitochondria (109). This implies that loss of cytoplasmic function is sufficient to induce parkinsonism. However, L166P more dramatically destabilizes the protein (116, 119–123). If both cytoplasmic and mitochondrial pools of the protein are depleted, then we cannot be sure that cytosolic DJ-1 is neuroprotective. In our hands, the effect of L166P in redirecting the protein to mitochondria was minor; both wild-type and mutant proteins could be found in either mitochondria or cytosol (119). As an aside, although the proteasome may be involved in degradation of L166P DJ-1, this does not imply that its function is related to parkin because the proteasome is a major degradation route for many mutant proteins.

Some progress has come from recent work showing that DJ-1 oxidation is directed toward a specific cysteine residue, C106 (124, 125). Oxidation converts the sulfhydryl group of C106 to a sulfonic or sulfinic acid, which correlates with the acidic pI shift of the protein. In our hands, DJ-1 is normally excluded from mitochondria in the majority of cultured cells but moves to the outer mitochondrial membrane under oxidative conditions, although this has not yet been confirmed in vivo. Correspondingly, DJ-1 protects cells against mitochondrial complex I inhibitors and other oxidative stresses (126, 127). Mutations at C106 that block the ability of DJ-1 to respond to oxidative stress exclude the protein from mitochondria and have a dominant negative effect on cell viability in response to mitochondrial damage (125).

These observations support the contention that loss of function of DJ-1 sensitizes neurons to oxidative stress (109). This would suggest that DJ-1 and parkin have effects on distinct neuronal survival pathways. However, others have reported that knockdown of DJ-1 sensitizes cells to both oxidative stress and proteasome inhibition or ER stress (126). Therefore, there is some clarification required to establish whether DJ-1 has a general effect on cell death or is specific to certain types of stresses. DJ-1 does not seem to have an effect on staurosporine-induced cell death, hence it does not suppress apoptosis per se (126).

Whether specific or general, the mechanism by which DJ-1 protects cells is not clear. One possibility is that DJ-1 acts as an antioxidant, scavenging hydrogen peroxide or other radical species (123, 127). Whether a protein that contains one readily oxidized cysteine residue would have a substantial effect in a cellular context where there are many other low-molecular-weight thiols is not known. Also, at least in our hands, DJ-1 becomes oxidized under conditions where cell death is not prominent. Perhaps oxidation of DJ-1 is a bellwether for the cell—warning of more damaging conditions to come and protecting cells before conditions become unmanageable.

What does DJ-1 do once oxidized? The recruitment to the outer mitochondrial membrane (125), if reproducible, might suggest that there is a suppression of pro-cell death pathways. Neuronal damage resulting from mitochondrial toxins such as MPTP involves some of these pathways [reviewed in (1)]. However, no binding partners at the outer mitochondrial membrane have been identified. Alternatively, DJ-1 might act as a chaperone (117), although this has been challenged (116). One could imagine that DJ-1 might play a role in suppressing neuronal damage by promoting the refolding of damaging proteins. DJ-1 has been found in association with intraneuronal inclusions formed by the microtubule-binding protein tau (66, 128) but only rarely labels Lewy bodies (129). As the neuropathology of DJ-1 cases is not yet reported, whether DJ-1-mediated disease includes the protein inclusion pathology resulting from lack of chaperone activity is not clear. DJ-1 may also be acting as a protease, but the physiological targets for this activity have not been identified. Returning to the hypothesis suggested by Bonifati and colleagues, DJ-1 might alter the transcriptional profile of cells by binding to GAPDH (113) or to components of the transcriptional machinery such as the SUMO-conjugating

enzyme PIASxα (130). Some of these binding partners also affect cell death; GAPDH promotes apoptosis in several models. Given these many possibilities, we can be sure only that DJ-1 is neuroprotective, arguably having something to do with mitochondrial function.

The third of the trio of recessive genes is PINK1 (110). Protein function is reasonably clear for PINK1, as it contains a serine/threonine-directed protein kinase domain. Human PINK1 and its mouse homologue had been cloned previously and possess authentic kinase activity against PINK1 itself (131, 132). PINK1 also has an N-terminal mitochondrial localization signal and transfected PINK1 is found in the mitochondria (110). A fusion protein with an N-terminal myc tag is present as a single band and thus is likely to be the preprotein. This preprotein is present in mitochondria, implying that PINK1 is imported and then cleaved; similar processes are well described for other mitochondrial matrix proteins. Although there is only one study to date, Valente and colleagues (110) reported that PINK1 protects cells against apoptosis induced by exposure to proteasome inhibitors. Critically, recessive mutations that are predicted to lack kinase activity were not neuroprotective. At the time of writing, there are some obvious gaps in our knowledge. The first question is whether this protection is specific to proteasome-induced stress or is more general. It is possible, for example, that PINK1 suppresses apoptotic signaling in response to several different stressors. Secondly, it will be critical to identify the physiological substrate for this mitochondrial kinase. Presumably, the substrate(s) plays some role in mitochondrial responses to stress, which might clarify whether the observed protection is specific or general.

PATHWAYS TO PARKINSONISM

The above discussion has deliberately separated the biochemistry of PD into two components: α-synuclein aggregation and cell death of susceptible neurons. As stated earlier, human genetics can indicate the proximal events in these diseases. Therefore, in this bipartite view of the disease process, dominant synuclein mutations account for the intracellular protein inclusion pathology, whereas recessive mutations tell us more about the pathways that lead to cell death. The remaining question is: Can we link these two sets of processes together experimentally or logically?

To discuss this question, I start with the most recent discoveries and work backwards. Although it is too early to be certain if our initial predictions about DJ-1 and PINK1 will hold true in vivo, both of these proteins protect cells against loss of mitochondrial function. It is unlikely that PINK1 and DJ-1 physically interact because they are probably on opposite sides of the mitochondrial membrane. The mitochondrial leader peptide of PINK1 should direct the kinase through the mitochondrial import machinery into the mitochondria. In contrast, when DJ-1 overlaps with mitochondria, it localizes to the outer mitochondrial surface (125). Therefore, if there is a common mitochondrial pathway for DJ-1 and PINK1, it is

at the level of the whole organelle. This is reasonable given the prominent roles that mitochondria play in determining cellular life or death. However, if we suppose that all these genes suppress cell death under many circumstances, it would be surprising that cell death is restricted to a subset of neurons and not a more general phenomenon. It is interesting that DJ-1 might be excluded from mitochondria unless cells are stressed (125). If the cell survival pathway is more important under specific conditions, then one might expect the pattern of cellular damage to be restricted to cells that undergo these stresses or are especially vulnerable to them.

If mitochondrial pathways are implicated in this scheme, we would predict that the neurons affected in recessive parkinsonism would be susceptible to mitochondrial damage. Although exposure to MPTP produces a Parkinsonian syndrome, toxicity is dependent on uptake by the dopamine transporter (74), so restriction to dopaminergic neurons is not surprising. However, rotenone also inhibits mitochondrial complex without requiring uptake via the dopamine transporter, so this is a test of whether some neurons are more sensitive than others to mitochondrial damage. Chronic administration of rotenone selectively damages dopaminergic neurons in the nigra (133), although some studies also report more generalized toxicity. The mechanism appears to involve free radicals (134); hence DJ-1 should protect neurons from rotenone toxicity. Therefore, mitochondrial complex I with attendant oxidative damage might account for some of the neuronal damage in Parkinsonian conditions, and we can relate this to loss of function mutations in DJ-1 and PINK1.

Parkin does not easily resolve into this scheme. Given the ubiquitylation activity of this enzyme, cell death is most obviously related to proteasome function. We can ask whether proteasome inhibition would be sufficient to induce cell death and whether such cell death would be restricted to the mosaic of cells susceptible in PD. This experiment was performed recently, and the patterns of cell death closely resemble those in sporadic PD (135). Proteasome inhibitors also preferentially affect catecholaminergic neurons in some (96), but not all (136), in vitro models. This implies that susceptible neurons in PD are linked by sensitivity to proteasome dysfunction.

Therefore, there are at least two pathways that can lead to Parkinsonian syndromes: mitochondrial and proteasomal. Logically, there are three ways to consider these two pathways. Firstly, perhaps each is sufficient to induce cell death and is independent of the other. In this scheme, the fact that some groups of neurons are affected by these stresses is coincidental, and the human syndromes are phenocopies of each other. A second possibility is that both mitochondrial and proteasomal damage are required for neuronal damage, having initially distinct events but converging on a later, common pathway. The third possibility is the neatest: The three genes mark a single pathway that we can connect in an ordered way.

However, it is hard to separate proteasomal from mitochondrial damage because they interact with each other. Addition of proteasome inhibitors increases the

sensitivity of catecholaminergic neurons to rotenone or MPTP in vitro (136). In this study, complex I inhibitors caused a decrease in proteasome activity. This may be the result of ATP depletion, as the ubiquitin-proteasome system is very heavily ATP dependent, or a consequence of oxidation, or both. Reciprocally, proteasome inhibitors have been reported to cause mitochondrial damage (137). Therefore, proteasomal and mitochondrial damage interact in both directions to converge on cell death as an outcome.

It is also possible that we have misunderstood one or more of these components. The evidence that parkin has an effect on mitochondria (138) is surprising for an E3 ligase with no mitochondrial substrates. The effects of parkin can be specific, as parkin is effective against apoptosis pathways that proceed through mitochondrial signaling but not other triggers (108). A more powerful example is when proteomics was used to examine the brains of parkin knockout mice. Although many proteins were present on two-dimensional gels, mitochondrial proteins were specifically represented (107). These observations led to the idea that mitochondria may be important in parkin disease as well as DJ-1 and PINK1, but specificity is unclear.

Such considerations become much more complex when we attempt to add α-synuclein into these schemes. The Dawson laboratory recently articulated these difficulties by proposing that there are two logical models (139). In the "unifying model," parkin and synuclein have differential effects on a common pathway, whereas in the "distinguishing model," PD and recessive parkinsonism have different pathways (139). This discussion centers on whether parkin plays an essential role in α-synuclein disease and vice versa. Alternatively stated, the problem is: What causes disease in these different conditions?

We can be sure that α-synuclein is causal in the PD/DLBD families, and it is likely that protein aggregation underscores the disease process. What happens downstream of protein aggregation to cause cell death is less clear. α-Synuclein has detrimental effects on both proteasomal [(57, 96) and references therein] and mitochondrial (140, 141) function. Interactions between mitochondria and proteasomal function were discussed above, but aggregated α-synuclein can inhibit the proteasome in vitro, suggesting that it might directly affect the ubiquitin proteasome system (142, 143). Adding to the confusion, mitochondrial damage may exacerbate α-synuclein aggregation, promoting the accumulation of the protein posttranslationally (100). This leads to many schemes of the pathogenesis of PD that evoke amplifying circles of mitochondrial damage, proteasomal dysfunction, and protein accumulation.

If α-synuclein affects both mitochondria and the proteasome and if mitochondrial/proteasomal genes cause parkinsonism, does this mean that α-synuclein mediates neuronal damage in the recessive diseases? α-Synuclein is a good candidate for being an endogenous stressor, as we know the wild-type protein can be toxic when present at high levels. One might imagine that lack of protective gene products (such as parkin, DJ-1, or PINK1) might make some neurons susceptible to the same causal agent at normal expression levels. An argument against α-synuclein involvement in cell death is the lack of obvious Lewy pathology in most parkin cases, although there are reported exceptions (73). However, if Lewy body

formation is not required for toxicity, as implied by A30P, then we might not need to see inclusion body pathology for α-synuclein to be toxic. Another argument is that parkinsonism is a component of diseases caused by other aggregating proteins, including tau mutations (144). There are several parallels between tau and α-synuclein. Both are intracellular proteins with a tendency to aggregate, perhaps coaggregating (145), and both proteins can cause cell death (146). Parkinsonism can be a component of the phenotype of patients' mutations in spinocerebellar ataxia genes (147), which are associated with aggregations of polyglutamine proteins. Therefore, α-synuclein is not the only brain protein that can aggregate and kill nigral cells, although it is one of the few that aggregates so readily in its wild-type form; tau is the other major one in brain.

In the absence of α-synuclein pathology in *parkin*, *DJ-1*, and *PINK1* cases, we cannot be certain about the causative agent in the same way as α-synuclein mutations. However, parkin can protect cells against α-synuclein toxicity. To my mind, this implies that the pathways triggered by the aggregating protein must converge at some point on the positive effects of parkin and other recessive gene products. A critical set of experiments will be to compare whether all three recessive parkinsonism genes are important in protecting against α-synuclein toxicity specifically or against toxic proteins in general. More importantly, we need to better define the relationships between the different recessive gene products and understand where their effects are specific and where they only coincidentally affect the same cellular processes.

Why should we care about the distinction between the concept of a single pathway and multiple roads to the same output, even if it is a tractable problem? The most practical benefit from really understanding the nature of cell death in PD and related disorders is the possibility of providing new therapeutic avenues. In this sense it is not critical whether events are early or late in the pathogenic process; each is an avenue for intervention. Identifying the earliest and most specific events that cause neuronal loss in these disorders might also indicate where to aim strategies with the highest level of specificity. No matter what the underlying cause of cell loss in parkin, DJ-1, or PINK1 disease, all three of these genes impact neuronal ability to survive in the face of stress, and I think it can be no coincidence that all three produce such a similar phenotype. I suspect that recessive genes tell us why neurons are damaged in Parkinsonian conditions but that our thinking about these is quite crude at this point. Certainly, we have yet to identify why different neuronal groups rely on these proteins more than others, which may be key to the problem of parkinsonism in many diseases.

CONCLUDING REMARKS

Identifying monogenic forms of PD has led us to a causative agent, α-synuclein. It is probable that causation is shared with sporadic PD in which intracellular aggregates of the same protein are found throughout the brain. Gene dosage is important, and this is correlated with increased toxicity of the protein at higher

expression levels. There is currently an unclear relationship between α-synuclein disease and recessive forms of parkinsonism. The challenges for the PD field are to describe in detail the routes that lead to toxicity in these different situations and answer whether, or not, these different pathogenic cascades overlap.

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NOTE ADDED IN PROOF

Since writing this chapter, the gene for the PARK8 locus has been cloned (151, 152). *LRRK2* is a large complex kinase and is currently not characterized. The protein has been termed Dardarin by Paisan-Ruiz et al. (151).

Annual Review of Biochemistry
Volume 74, 2005

CONTENTS

FROM PROTEIN SYNTHESIS TO GENETIC INSERTION, *Paul Zamecnik* 1

THE BIOCHEMISTRY OF PARKINSON'S DISEASE, *Mark R. Cookson* 29

APPLICATIONS OF DNA MICROARRAYS IN BIOLOGY, *Roland B. Stoughton* 53

ZONA PELLUCIDA DOMAIN PROTEINS, *Luca Jovine, Costel C. Darie, Eveline S. Litscher, and Paul M. Wassarman* 83

PROLINE HYDROXYLATION AND GENE EXPRESSION, *William G. Kaelin Jr.* 115

STRUCTURAL INSIGHTS INTO TRANSLATIONAL FIDELITY, *James M. Ogle and V. Ramakrishnan* 129

ORIGINS OF THE GENETIC CODE: THE ESCAPED TRIPLET THEORY, *Michael Yarus, J. Gregory Caporaso, and Rob Knight* 179

AN ABUNDANCE OF RNA REGULATORS, *Gisela Storz, Shoshy Altuvia, and Karen M. Wassarman* 199

MEMBRANE-ASSOCIATED GUANYLATE KINASES REGULATE ADHESION AND PLASTICITY AT CELL JUNCTIONS, *Lars Funke, Srikanth Dakoji, and David S. Bredt* 219

STRUCTURE, FUNCTION, AND FORMATION OF BIOLOGICAL IRON-SULFUR CLUSTERS, *Deborah C. Johnson, Dennis R. Dean, Archer D. Smith, and Michael K. Johnson* 247

CELLULAR DNA REPLICASES: COMPONENTS AND DYNAMICS AT THE REPLICATION FORK, *Aaron Johnson and Mike O'Donnell* 283

EUKARYOTIC TRANSLESION SYNTHESIS DNA POLYMERASES: SPECIFICITY OF STRUCTURE AND FUNCTION, *Satya Prakash, Robert E. Johnson, and Louise Prakash* 317

NOD-LRR PROTEINS: ROLE IN HOST-MICROBIAL INTERACTIONS AND INFLAMMATORY DISEASE, *Naohiro Inohara, Mathias Chamaillard, Christine McDonald, and Gabriel Nuñez* 355

REGULATION OF PROTEIN FUNCTION BY GLYCOSAMINOGLYCANS—AS EXEMPLIFIED BY CHEMOKINES, *T.M. Handel, Z. Johnson, S.E. Crown, E.K. Lau, M. Sweeney, and A.E. Proudfoot* 385

STRUCTURE AND FUNCTION OF FATTY ACID AMIDE HYDROLASE, *Michele K. McKinney and Benjamin F. Cravatt* 411

NONTEMPLATE-DEPENDENT POLYMERIZATION PROCESSES: POLYHYDROXYALKANOATE SYNTHASES AS A PARADIGM, *JoAnne Stubbe, Jiamin Tian, Aimin He, Anthony J. Sinskey, Adam G. Lawrence, and Pinghua Liu* 433

EUKARYOTIC CYTOSINE METHYLTRANSFERASES, *Mary Grace Goll and Timothy H. Bestor* 481

MONITORING ENERGY BALANCE: METABOLITES OF FATTY ACID SYNTHESIS AS HYPOTHALAMIC SENSORS, *Paul Dowell, Zhiyuan Hu, and M. Daniel Lane* 515

STRUCTURE AND PHYSIOLOGIC FUNCTION OF THE LOW-DENSITY LIPOPROTEIN RECEPTOR, *Hyesung Jeon and Stephen C. Blacklow* 535

COPPER-ZINC SUPEROXIDE DISMUTASE AND AMYOTROPHIC LATERAL SCLEROSIS, *Joan Selverstone Valentine, Peter A. Doucette, and Soshanna Zittin Potter* 563

THE STRUCTURE AND FUNCTION OF SMC AND KLEISIN COMPLEXES, *Kim Nasmyth and Christian H. Haering* 595

ANTIBIOTICS TARGETING RIBOSOMES: RESISTANCE, SELECTIVITY, SYNERGISM, AND CELLULAR REGULATION, *Ada Yonath* 649

DNA MISMATCH REPAIR, *Thomas A. Kunkel and Dorothy A. Erie* 681

GENE THERAPY: TWENTY-FIRST CENTURY MEDICINE, *Inder M. Verma and Matthew D. Weitzman* 711

THE MAMMALIAN UNFOLDED PROTEIN RESPONSE, *Martin Schröder and Randal J. Kaufman* 739

THE STRUCTURAL BIOLOGY OF TYPE II FATTY ACID BIOSYNTHESIS, *Stephen W. White, Jie Zheng, Yong-Mei Zhang, and Charles O. Rock* 791

STRUCTURAL STUDIES BY ELECTRON TOMOGRAPHY: FROM CELLS TO MOLECULES, *Vladan Lučić, Friedrich Förster, and Wolfgang Baumeister* 833

PROTEIN FAMILIES AND THEIR EVOLUTION—A STRUCTURAL PERSPECTIVE, *Christine A. Orengo and Janet M. Thornton* 867