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Proteomic Approach to Studying Parkinson's Disease

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Abstract

Parkinson's disease is a common age-related neurodegenerative disease characterized pathologically by a loss of dopaminergic neurons in the substantia nigra with resultant depletion of striatal dopamine and presence of Lewy bodies in the remaining neurons. The Lewy body contains numerous functional and structural proteins, including α-synuclein and ubiquitin; aggregation of α-synuclein is thought to be important in Lewy body formation as well as neurodegeneration, although the detailed mechanisms remain to be defined. Increasing evidence has suggested that mitochondrial dysfunction, increased oxidative stress, and dysfunction of the ubiquitin-proteasome system may be involved in α-synuclein aggregation, Lewy body formation, and neurodegeneration. However, how these processes are related to each other is not fully understood, given that there are Parkinsonian animal models as well as human diseases with significant nigral neurodegeneration regardless of whether Lewy bodies form or not. This review summarizes the current related research fields and proposes a proteomic approach to investigate the mechanisms that may dictate α-synuclein aggregation, Lewy body formation, and neurodegeneration.

Index Entries: Parkinson's disease; α-synuclein; Lewy body; proteomics.

Parkinson's disease (PD) is characterized by a loss of dopaminergic neurons from the substantia nigra (SN), depletion of striatal dopamine (DA), and the presence of intraneuronal inclusions called Lewy bodies *(1)*. The cause of neuronal death underlying PD is unknown, but appears to involve the intertwined processes of aging, genetic susceptibility, and environmental exposures *(2–5)*. One of the central issues is how these factors might interact with each other to mediate Lewy body formation and neurodegeneration. The recent description of the failure of proteasomal function in PD suggests that various etiological factors may converge and start with proteolytic stress, i.e., a state in which the levels of unwanted proteins exceed the capacity for clearance due to increased protein production and/or inadequate proteolysis. In some

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familial PD, mutations in the genes encoding α-synuclein, parkin, and ubiquitin C-terminal hydrolase L1 (UCH-L1) are associated with protein accumulation and Lewy body formation in the substantia nigra (SN), locus ceruleus, and cerebral cortex *(6–8).* In sporadic PD, the levels of oxidatively damaged proteins and protein aggregation are elevated in the SN, and are probably secondary to mitochondrial dysfunction observed in these patients *(9–11).* In addition, recent studies have revealed structural and functional defects in 26/20S proteasomes in the SN in sporadic PD *(12–14).*

On the other hand, there are human diseases with nigrostriatal degeneration with α-synuclein aggregation but without Lewy body formation. Furthermore, while various Parkinsonian models all demonstrate relatively selective nigrostriatal degeneration, they differ substantially in the ability in producing Lewy body-like cytoplasmic inclusions *(15–17).* The molecular mechanisms underlying these phenomena are not known, but likely relate, at least partially, to proteins interacting with α-synuclein, whose aggregation into soluble and insoluble aggregates is believed to be crucial in dopaminergic neurodegeneration *(18–25).* Currently, extensive research is being conducted in characterizing these protein–protein interactions, and there have been numerous advances in technology in these areas as well. Hence, this review is designed to briefly summarize the current research fields and discuss a new technique, proteomics, which may contribute to PD research tremendously in the years to come.

UPS, α-Synuclein, Lewy Bodies, and Neurodegeneration

The ubiquitin proteasome system (UPS) is the primary biochemical pathway responsible for nonlysosomal degradation of normal and abnormal intracellular proteins, and its failure leads to protein accumulation and cell death *(26–28).* Proteasomes are multicatalytic proteases found in the cytoplasm, endoplasmic reticulum, perinuclear region, and nucleus of eukaryotic cells *(27).* The 20S proteasome is an assembly of two outer heptameric rings of α subunits and two inner heptameric rings of β subunits that are stacked axially to form a hollow cylindrical structure in which proteolysis occurs *(27,29).* Binding of the multisubunit intracellular proteasome activators, PA700 and PA28, to the 20S proteasome form more active complexes known as the 26S proteasome and the activated 20S proteasome, respectively *(27,29).* While proteins that have been marked for proteolysis by labeling with a polyubiquitin chain are degraded by the 26S proteasome in an ATP-dependent manner, nonubiquitinated proteins, short peptides, short-lived regulatory proteins, and some oxidatively damaged proteins are degraded by 20S proteasomes via a mechanism that is independent of ATP *(27,29–33).*

Mutations in the genes encoding two enzymes of the UPS, parkin and ubiquitin C-terminal hydrolase L1 (UCH-L1), are associated with cases of familial PD *(7,8).* The proposed links of these two proteins with UPS are illustrated in Fig. 1. In addition, the gene that encodes α-synuclein also appears to link the UPS to PD. α-Synuclein is a neuronal protein of unknown function, which is normally localized to the presynaptic terminals in mammalian brains. Pathologically, it has been found to be a major constituent of Lewy bodies in PD and dementia with Lewy bodies (DLB) *(1),* as well as glial cytoplasmic inclusions in multiple system atrophy (MSA) *(34)* and dystrophic neurites in Hallervorden-Spatz disease *(35).* The discovery of cosegregation of two types of missense mutations in the α-synuclein gene (A30P *(36)* and A53T *(6)*) with the clinical manifestations of PD in autosomal-dominantly inherited familial PD strongly implicates the role of α-synuclein in neuronal degeneration. The mechanism whereby mutations of α-synuclein lead to neuronal degeneration is not completely understood; mutated α-synuclein may form oligomers of α-synuclein or insoluble fibrils such as in Lewy bodies that are refractory to the UPS-mediated proteolysis, ultimately

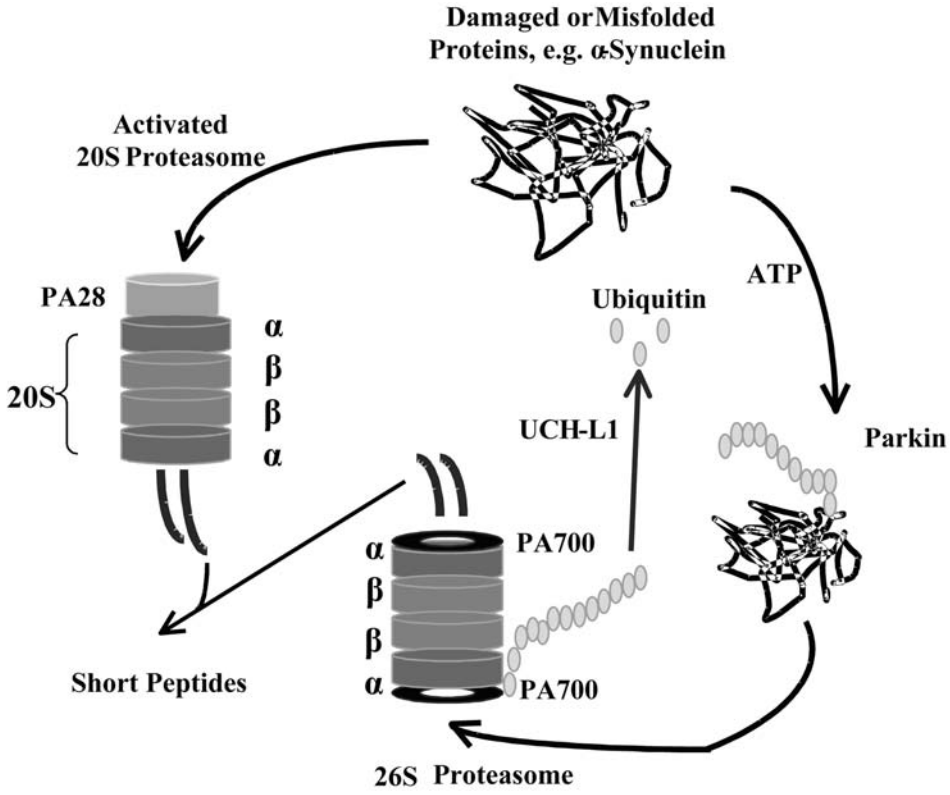

Fig. 1. Diagrams of some important elements associated with the UPS system in relation to PD. In essence, damaged and unfolded or misfolded proteins, whether derived from genetic mutation, aging, or environmental exposures, are either directly degraded by 20S proteasome independent on ATP or by 26S proteasome via ubiquitination pathway that is dependent on ATP. Parkin, an E3 ligase, is responsible to recruit the substrate, and the polyubiquitin chain is released by the PA 700 and further degraded to free ubiquitin by UCH-L1.

leading to cell death *(25).* In fact, aggregation of abnormal α-synuclein is believed to be pivotal in the pathogenesis of PD *(18–25).* Evidence to support this hypothesis includes: (a) α-synuclein is a substrate for the UPS *(8,37)* and recent data indicate that the 20S proteasome is primarily responsible for α-synuclein metabolism and degradation *(37);* (b) mutations in α-synuclein cause the protein to misfold and aggregate, resist proteolysis, and inhibit proteasomal function *(38);* (c) misfolded and/or aggregated α-synuclein directly induces mitochondrial dysfunction and increases oxidative stress *(39,40),* the two most consistent findings in PD patients; and (d) α-synuclein-null mice display functional deficits in the nigrostriatal DA system *(41)* and are resistant to MPTP-induced degeneration of dopaminergic neurons, indicating that normal α-synuclein function is important to dopaminergic neuron viability *(42).* The results in transgenic *Drosophila,* mouse, and rat models of PD involving manipulation of α-synuclein are intriguing: overexpression of wild-type α-synuclein in all three models recapitulates essential features of the human disease, including formation of Lewy body-like inclusions *(43–45).* However, no significant difference in the extent of nigral damage is found between *Drosophila* or rats expressing wild-type and

mutated α-synuclein *(44–46).* More recent investigation seems to suggest that, mutated or not, α-synuclein can aggregate and mediate neurodegeneration as long as a critical threshold of α-synuclein level is crossed *(47).*

While mutations in α-synuclein, parkin, and UCH-L1 do not occur in sporadic PD, these proteins are present in Lewy bodies of sporadic PD patients *(48,49),* suggesting that genetic vulnerability and aging or environmental exposure, two major factors in the development of sporadic PD, may converge at dysfunction of the UPS, leading to formation of Lewy bodies as well as dopaminergic neurodegeneration *(44).* In fact, experimental data have demonstrated that, in comparison to age-matched controls, α subunits (but not β subunits) of 26/20S proteasomes are lost within dopaminergic neurons and 20S proteasomal enzymatic activities are impaired in the SN compacta (SNc) in sporadic PD *(12,13,49).* In addition, the levels of PA700 and PA28, proteasomal activators, are reduced in the SNc in PD *(13).* Furthermore, inhibition of proteasomal function alone appears to be sufficient to bring about nigral degeneration with formation of Lewy body-like inclusions in rats *(50).* The cause of defects in 26/20S proteasomes in sporadic PD is at present unknown, but it could be acquired mutations in one or several of the genes that encode for α subunits or other components of 26/20S proteasomes. Alternatively, proteasomal dysfunction could result from direct inhibition by environmental toxicants *(51)* or by oxidant-mediated damage to proteins. Indeed, α subunits of 26/20S proteasomes are selectively vulnerable to oxidative stress *(52).*

The precise mechanisms underlying UPS dysfunction, aggregation of α-synuclein or other proteins, and cell death are currently not understood. The key players may be the proteins that interact with these components, including heat shock proteins (HSPs). HSPs are critical elements of the cellular response to unfolded proteins and are involved in promoting proper protein folding and preventing aggregation, as well as promoting ubiquitination and degradation of misfolded proteins *(53).* Failure in one or both of these pathways can result in accumulation of misfolded proteins with consequent cellular toxicity. Directed expression of the molecular chaperone hsp70 prevents dopaminergic neuronal loss in *Drosophila,* and interference with endogenous chaperone activity accelerates α-synuclein toxicity *(46).* Similar results have been found in the Huntington's model, where chaperon suppresses cellular toxicity of huntingtin *(54).* More recently Auluck and Bonini demonstrated that geldanamycin, a naturally occurring benzoquinone ansamycin that specifically binds to and interferes with the activity of the molecular chaperone hsp90, completely prevents α-synuclein-induced toxicity in *Drosophila (55).* Interestingly, coexpression of hsp70 in *Drosophila* prevents dopaminergic neuronal loss induced by α-synuclein transfection without affecting formation of Lewy body-like inclusions *(46),* suggesting that pre-Lewy body steps in α-synuclein aggregation are the real toxic species. This concept is further supported by the experiments showing that soluble α-synuclein oligomers *(56–60)* are more toxic than insoluble fibrils *(61,62).* With quantitative proteomics, our recent experiments (to be published) demonstrated that α-synuclein was associated not only with hsp70 and 90, but also with numerous other proteins (more than 200), including those related to the UPS and mitochondrial function as well as oxidative stress in dopaminergic cells when treated with Parkinsonian toxicants, i.e., rotenone and 1-methyl-4-phenylpyridine (MPP^+), the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Of note, unlike rotenone, MPP^+ does not produce Lewy body-like cytoplasmic inclusions (see details below). Interestingly, the composition of proteins associated with α-synuclein was different between cells with and without Lewy body-like cytoplasmic inclusions, even though both toxicants produced α-synuclein aggregation and comparable neurotoxicity. Finally, transfection of hsp70 attenuated not only rotenone-mediated toxicity, but also α- synuclein aggregation and forma-

tion of Lewy body-like inclusions. These results not only support the hypothesis that proteins associated with α-synuclein play important roles in neurotoxicity, but also indicate that proteins interacting with α-synuclein may dictate whether α-synuclein aggregates and Lewy bodies form.

In summary, there is clear evidence of UPS dysfunction in both familial and sporadic PD, which may be responsible, at least partially, for aggregation of α-synuclein and Lewy body formation. However, it is not clear what causes α-synuclein to aggregate and whether its aggregation into Lewy bodies is important in dopaminergic neurodegeneration in PD. The key factors that determine these processes may be the proteins that interact with α-synuclein.

Interaction Among Oxidative Stress, Mitochondrial Dysfunction, and UPS

We, as well as others, have demonstrated that sporadic PD is associated with significant oxidative damage in the SN *(9,10,63,64).* The sources for production of reactive oxygen species (ROS) in PD have not been characterized fully, although damaged (leaking) mitochondria appear to be one of the major causes *(65),* given that they utilize more than 90% oxygen delivered to cells *(66).* Several groups of investigators have reported mitochondrial dysfunction in PD patients *(67–71).* Mitochondrial oxidative phosphorylation is carried out by five protein-lipid enzyme complexes located in the mitochondrial inner membrane *(72).* Among those proteins, 13 peptides are derived from mitochondrial DNA (mtDNA) *(73),* a small covalently closed circular DNA molecule *(65).* Compelling evidence has suggested that an alteration in mtDNA may be particularly important in PD since cybrids, a cell line devoid of mitochondria, that express PD mtDNA show reductions in complex-I activity *(74).* While most mitochondrial PD studies have focused on complex-I activities, other mitochondrial complexes may also be affected, and mitochondrial dysfunction may not be restricted to the SN *(68,69,75).* In fact, we recently found that mitochondrial DNA damage in PD is widespread without restriction to any given regions of mtDNA or regions of brain *(76,77).* Furthermore, besides the 13 peptides coded by mtDNA for the respiration complex, there are hundreds of other protein peptides in mitochondria derived from nuclear DNA *(78,79).* It is likely, therefore, that a defect in any components of mitochondrial respiratory chain or in other gene products involved in normal mitochondrial function, whether inherited or acquired, may compromise mitochondrial function and potentially culminate in dopaminergic neurodegeneration in PD. Indeed, contemporary advances in mitochondrial biology have revealed that, far from being merely a supplier of ATP for the cell, mitochondria play significant roles in redox cell signaling *(80),* apoptotic cell death *(81),* and cellular homeostasis *(80).*

The cause of mitochondrial dysfunction in PD has not been defined clearly, although increased oxidative stress may play an important role *(9,10,63).* It should be noted that, as mentioned earlier, mitochondrial dysfunction also enhances oxidative stress *(82–86),* and it remains to be determined whether mitochondrial dysfunction is a cause or a consequence of increased oxidative stress in PD, or whether these two factors interact in a pathologically reinforcing cycle. In addition, it is not entirely clear why nigral neurons are selectively vulnerable to neurodegeneration and Lewy bodies tend to form in the SN while mitochondrial dysfunction is not restricted to this region in PD. The current hypothesis to explain this phenomenon centers on the catecholaminergic neurotransmitters contained in these neurons as additional sources of oxidative stress *(87–89).* In fact, we as well as others have shown that products of catechol oxidation enhance oxidative stress, inhibit mitochondrial function, and mediate neuronal death in experimental model systems *(90–93).* This increased oxidative stress may directly inhibit proteasomal function or produce damaged proteins

(94), including α-synuclein *(95–97),* that are resistant to proteasomal degradation *(1,9,11,98).* Finally, catechols may stabilize protein aggregates directly *(99,100).* Hence, catechols contained in these neurons may not only be responsible for their vulnerability to neurodegeneration, but may also explain why protein aggregates and Lewy body formation preferentially occur there.

The relationship between mitochondrial and proteasomal function is apparent, as ATP is required in protein ubiquitination, degradation, deubiquitination, and the regulation of these processes. Thus, it is proposed that mitochondrial dysfunction will contribute to UPS dysfunction in PD, particularly 26S proteasomal function *(31).* Conversely, proteasomal function also influences mitochondrial function. For example, mutation in a proteasomal gene in yeast, RPN11/MPR1, produces mitochondrial defects with altered mitochondrial morphology *(101).* Also, mutation in another proteasomal gene in yeast, ynt1-1, suppresses mitochondrial defects arising from a mutation in a mitochondrial AAA protease gene, yme1-1 *(102).* Furthermore, proteasomes can influence cell viability by affecting the half-life of Bcl-2 *(103)* or smac *(104),* mitochondrial proteins related to apoptosis. Finally, a close relationship between proteasomal and mitochondrial function can also be seen indirectly in experiments showing that misfolded and aggregated α-synuclein induces mitochondrial dysfunction *(39),* and that mutant α-synuclein-mediated mitochondrial abnormality and apoptotic cell death can be enhanced significantly by an exogenous proteasome inhibitor *(38).*

To sum up, the significance of mitochondrial dysfunction in PD is not just associated with reduced ATP production; it can also increase oxidative stress and influence proteasomal function. Conversely, oxidative stress and proteasomal function may negatively affect mitochondrial function as well. It is likely that it is these interrelated processes that ultimately contribute to protein aggregation, Lewy body formation, and cell death in PD.

Lewy Bodies and Neurodegeneration in a Few Parkinsonian Animal Models and Human Nigral Degenerative Diseases

As mentioned earlier, overexpression of normal or mutant human α-synuclein in nonrodent species can generate genetic PD models. In flies, which contain no endogenous α-synuclein, α-synuclein expression causes age-related depletion of dopamine neurons. This is proposed to be related to the abnormal aggregation of α-synuclein, and can be prevented by concomitant expression of a molecular chaperone hsp70 *(44,46).* In rodents, however, despite various promoters, α-synuclein transgenes and viral vectors have been tried to generate α-synuclein transgenic mice or Parkinsonian model in rats, the results are variable. In addition, none of these animals show a true Parkinsonian condition that includes Lewy bodies *(105–107).* It is possible that higher and more consistent levels of transgene expression will be needed to create a more useful transgenic mouse or rat model, as a recent observation suggesting that even normal α-synuclein, when present in excessive amount, can induce PD *(47).* Nonetheless, it is important to stress that there is no known genetic mutation in the vast majority of PD patients; hence, in the remainder of discussion, we will be focusing on a few animal models that are used to mimic sporadic PD.

Several mitochondrial toxicants have been used to generate Parkinsonian animal models with preferential striatal DA depletion and increased oxidative stress. Greenamyre and colleagues showed that systemic administration of the herbicide rotenone, a potent inhibitor of mitochondrial complex I associated with increased ROS production, provokes selective nigral degeneration reminiscent of PD with formation of Lewy body-like inclusions *(108,109).* The authors then confirmed that formation of Lewy body-like inclusions is directly linked to complex I inhibition in an in vitro model *(24).* In line with these observations, others have demonstrated that formation of cytosolic inclu-

sion bodies induced by rotenone can be reversed by removing rotenone from the culture medium with associated restoration of mitochondrial function *(110).* Finally, the importance of complex I inhibition by rotenone in neurodegeneration has been further substantiated by an elegant experiment showing that transfection of a single-subunit NADH dehydrogenase of *Saccharomyces cerevisiae,* Ndi1P, can work as a replacement for complex I in mammalian cells and confer resistance of cells to rotenone-mediated toxicity *(111).*

On the other hand, rotenone is not the first complex-I inhibitor to be associated with selective nigral neuronal degeneration and increased oxidative stress, as this is the proposed mechanism of action of MPP^+, the active metabolite of another neurotoxin, MPTP, which produces oxidative stress and causes a Parkinson-like syndrome in humans and various animals without formation of true Lewy bodies *(112–119).* Furthermore, although MPTP/MPP^+ does not produce true Lewy body-like inclusions *(117),* it increases α-synuclein expression *(120,121),* oxidatively modifies α-synuclein *(122),* and promotes cytoplasmic α-synuclein aggregation in the nigral neurons of animals *(123,124).* Interestingly, proteasomal inhibition with lactacystin does not apparently enhance MPP^+-mediated toxicity in cell cultures *(125).* What determines whether α-synuclein aggregation leads to Lewy body formation is not known, but several important differences should be noted between the effects of these two toxicants. First, rotenone causes complex-I inhibition throughout the brain, but degeneration is largely limited to the SN and locus coeruleus *(108),* whereas MPP^+ appears to be more targeted, and is selectively taken up by dopaminergic nerve terminals *(126,127).* Second, rotenone depletes striatal DA slowly over weeks, whereas depletion of striatal DA by MPTP/MPP^+ reaches a maximum in about 3–7 d. In fact, some observers have already reported that, despite the fact that there are no true Lewy bodies at any stages of this model, some kind of cytoplasmic aggregates can be seen in the SN of animals weeks or months after treatment with MPTP *(123,124).* Finally, rats are very resistant to MPTP-induced toxicity, unlike rotenone toxicity, when treated systemically. Although the reason for this is controversial *(128),* it is thought to be attributable at least in part to the inability of rats treated systemically with MPTP to accumulate significant amounts of MPP^+ in the dopaminergic system. Direct injection of MPP^+ into the SN *(116)* or medial forebrain bundle *(129),* or intrastriatal perfusion *(130)* at commonly used doses, does lead to massive DA loss in the striatum, but it also produces toxicity to adjacent brain structures, as indicated by a significant reduction in striatal γ-aminobutyrate (GABA) content *(131)* and absence of amphetamine-induced ipsilateral bias rotation *(132).* Of note, intraventricular injection of MPP^+ *(133,134)* appears to reduce the nonspecific neurotoxicity of MPP^+, indicating that nonselective neurotoxicity associated with cerebral injection of MPP^+ may be related to higher concentrations of MPP^+ injected locally.

Another neurotoxicant that has been widely used to generate a Parkinsonian model is 6-hydroxydopamine (6-OHDA), a hydroxylated analog of the natural DA neurotransmitter. Like MPP^+, 6-OHDA is also selectively taken up by the dopaminergic system and produces a pattern of cell death evocative of PD, but does not lead to formation of Lewy body-like cytoplasmic inclusions *(115,135–141).* In experimental models, when 6-OHDA is injected unilaterally into the striatum, the SN, or the medial forebrain bundle, especially in rats, it destroys nigral dopaminergic neurons and depletes the striatum of DA neurotransmitter, reaching a maximum within 5–7 d, thus reproducing the physiopathological features responsible for motor impairments in PD *(135).* This unilateral lesion of the nigrostriatal dopaminergic neurons by 6-OHDA initiates a characteristic circling behavior, which can be quantified in a rotation-box: DA-receptor agonists such as L-dopa lead to contralateral rotation, while DA-releasing substances such as amphetamine lead to ipsilateral rotation *(142).* It is thought that 6-OHDA induces catecholaminergic cell death by three main mechanisms: ROS generation by intra- or extracellular auto-oxidation *(87,88,143,144),*

H_2O_2 formation induced by MAO activity *(145),* and direct inhibition of the mitochondrial respiratory chain, including complex I *(146–148).* Interestingly, some evidence suggests that 6-OHDA may be a physiological endogenous neurotoxin, as several studies reported the presence of 6-OHDA in both rat and human brains *(149,150).* More recently, experimental data have demonstrated that although 6-OHDA does not produce true Lewy bodies, it increases levels of ubiquitin-conjugated proteins, and its toxicity is enhanced by proteasomal inhibitors *(151).* Unlike MPP^+, however, α-synuclein aggregation has not been reported even after weeks of treatment with 6-OHDA. Again, what distinguishes rotenone or MPP^+ from 6-OHDA is not known. One of the possibilities is that products of autooxidation of 6-OHDA or its metabolites may crosslink proteins directly, but how this process might prevent α-synuclein aggregation or Lewy body formation is not understood.

Parkinsonian animal models, though similar, are not identical to PD in human. For instance, none of the models including rotenone *(108,109)* shows classic histological features of Lewy bodies in human PD. Also, none of the models, including monkeys treated with MPTP *(152),* exhibit the typical "pill-rolling" type of resting tremor, one of the cardinal signs of PD. On the other hand, Lewy bodies are found not only in PD but also in dementia with Lewy body disease (DLB), which is distinctly different from PD both clinically and pathologically, primarily because of the widespread presence of Lewy bodies in other brain regions *(153).* Furthermore, α-synuclein aggregation (synucleinopathy) has been seen in multiple-system atrophy (MSA) and a few other degenerative diseases not associated with Lewy body formation. Detailed descriptions of these diseases are beyond the scope of this brief background review, but it needs to be pointed out that there are no animal models currently for any of these diseases, including DLB and MSA. Consequently, the pathological differences between PD and these diseases currently can be studied only with human tissue directly. To perform human tissue studies, it is best to use autopsy materials from subjects who have been carefully evaluated clinically before death and whose final diagnoses are confirmed by pathological examination. The latter is particularly important, given that none of these diseases can be definitively diagnosed clinically.

To summarize, although all three Parkinsonian toxicants produce relatively selective nigrostriatal damage, they differ in their ability to produce Lewy body-like inclusions. There are also human nigral degenerative diseases with and without Lewy body formation. Since α-synuclein appears to play a major role in neurodegeneration in PD, and increasing evidence has suggested that proteins that interact with α-synuclein may influence α-synuclein aggregation, Lewy body formation, and cell death significantly, it will be extremely helpful to compare all the proteins that interact with α-synuclein in these animal models or human diseases so that our knowledge on the pathogenesis of α-synuclein aggregation, Lewy body formation, and neurodegeneration can be advanced.

Proteomics as a Tool for Identifying Proteins and Mapping Protein–Protein Interactions

Mass spectrometry (MS) has revolutionized the biological sciences since the development of matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) in the late 1980s *(154),* facilitating the emergence of a new discipline called proteomics. The most common method of quantitative proteome analysis is comparative two-dimensional gel (2-D) electrophoretic separation of proteins from two different biological states, followed by MS mass mapping or tandem MS (MS/MS) to identify the proteins of interest. Two-dimensional electrophoresis separation stands out by its remarkable resolving power and, lately, ease of use for quantitative analysis, which have increased sensitivity, reproducibility, and throughput of proteome analysis *(79,155,156).* In addition, 2-D electrophoresis is highly suitable for the study of

post-translational modifications because isoforms are easily resolved. However, the 2-D electrophoresis approach is not without limitations *(157).* Specifically, in the case of quantification, one stained spot may contain more than one protein, only one of which is changing between the biological states studied, making it difficult to know for certain which protein is changing. More importantly, there are problems arising from chemical heterogeneity, exemplified by the classes of poorly water-soluble proteins such as membranous and nuclear proteins, which are very hard to analyze with 2-D electrophoresis. Although prefractionation strategies and progress in technologies, e.g., introduction of pH gradient gels, can overcome some of these problems, and hence improve the coverage of the total proteome of a cell, it seems quite obvious that these issues will remain major limitations of 2-D-based approaches *(158).*

These limitations of 2-D electrophoresis have prompted several research groups to introduce gel electrophoresis-free approaches; some of the pioneers of these techniques are our colleagues and collaborators at the Institute for Systems Biology in Seattle *(159–162).* In brief, the total protein mixture is digested, typically by trypsin, and then loaded on a multidimensional peptide liquid chromatographic (LC) separation, interfaced in line with an MS/MS spectrometer. This technique appears to be a much better proteomic screen than 2-D electrophoresis, since about 1000 proteins may be identified and, if carried out in conjunction with the isotope-coded affinity tag (ICAT) method *(159),* quantified in a single experiment. The ICAT probe consists of a biotin tag, a linker, and an iodoacetamide handle. The linker typically contains either a light (^{12}C) or a heavy (^{13}C) version, forming two probes with indistinguishable chemical behavior but with a mass difference of 9 Da. Control and experimental protein extracts are labeled with either a light or a heavy ICAT reagent at cysteine, then pooled and digested to peptides with trypsin. The cysteine-containing peptides are purified by strong cation exchange and avidin affinity chromatography and then fragmented by a tandem mass spectrometer after separation by an on-line microcapillary HPLC. Combining the heavy and light protein mixtures immediately after labeling significantly minimizes sample-handling variations between treatment and control. This approach (diagrammed in Fig. 2) has proven to be reproducible and sensitive by many research

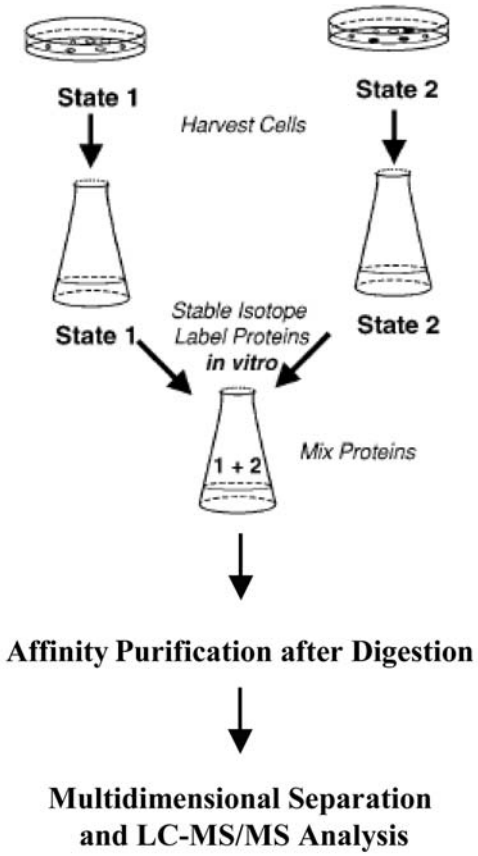

Fig. 2. ICAT Assay. To compare relative, not absolute, amounts of proteins between two different experimental states by proteolysis and mass spectrometric analysis, stable isotopes, typically ^{12}C and ^{13}C, are introduced into proteins isolated from two experimental conditions. After labeling, proteins are usually digested with trypsin, and peptides are separated by liquid chromatography (often multidimensional). Proteins are identified from peptides (e.g., by mass mapping or tandem MS) and quantified by normalization of the MS response of one labeled peptide from state 1 to the chemically identical but stable isotope labeled peptide from state 2. This figure is modified based on ref. *163.*

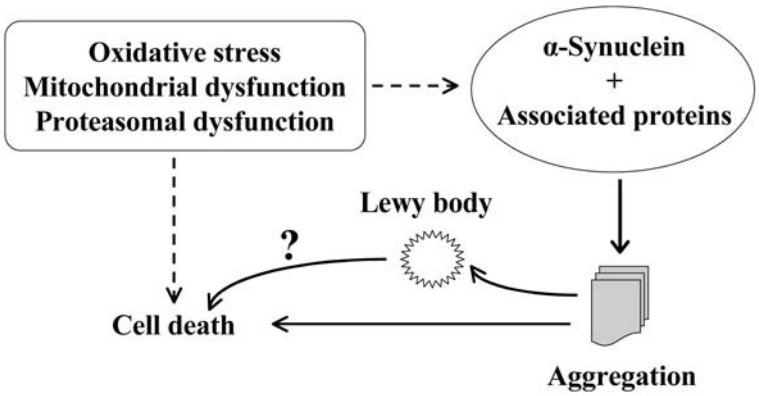

Fig. 3. Interrelated processes of increased oxidative stress, mitochondrial inhibition, and proteasomal dysfunction are thought to promote α-synuclein aggregation by mechanisms yet to be defined, thereby contributing to Lewy body formation and neurodegeneration in PD. Of note, a direct role for Lewy body formation in neurodegeneration is still controversial.

groups, although it must be recognized that some proteins including α-synuclein do not have any cysteine *(158).* The ICAT method strives to achieve high proteome coverage by sacrificing high individual protein sequence coverage. Thus, thorough analysis of post-translational modification is problematic. Recently, however, new approaches have been introduced to overcome some of these problems, e.g., linkers tagged on different amino acids and assays that can detect phosphorylated proteins; however, most of these approaches are currently either not fully established yet or suffer from quantitative drawbacks.

Despite the advances in proteomic techniques, complete global proteome analysis of complex protein mixtures, e.g., brain homogenates, will remain difficult in the near future largely because current proteomic technologies are biased toward abundant proteins. This limitation arises from the "top down" mode of MS operation, i.e., MS preferentially or exclusively analyzes the most abundant proteins. There are a few strategies that can be used to mitigate this problem, including the analysis of functional multiprotein complexes such as the ribosome *(161),* spliceosome *(164),* and nuclear pore complex *(165),* or organelles, such as mitochondria *(166)* and nuclei *(167).* Alternatively, proteins that contain common distinguishing structural features, such as phosphate ester groups *(168)* or cysteine residues *(169)* or that have the ability to specifically bind to certain compounds *(170,171)* have been selectively enriched prior to MS analysis. These strategies have in common that they focus on the in-depth (ideally complete) analysis of sub-proteomes of rich biological context, thus minimizing the repeated analyses of abundantly expressed proteins.

Briefly, proteomics is a powerful tool that can identify hundreds and thousands of proteins in a single experiment; however, extensive analysis of a particular proteome requires simplification of the specimen. In PD research, one obvious target that can be focused on with proteomic analysis is α-synuclein-associated protein complex, since α-synuclein aggregation is critical not only in formation of Lewy bodies but also in neurodegeneration. Other alternatives would include mitochondria and proteasomes, both of which are important in PD pathogenesis.

Conclusion

As indicated in Fig. 3, many encouraging results show that oxidative stress and dysfunction of mitochondrial and proteasomal func-

tion play a pivotal role in the pathogenesis of PD. Key questions related to the role of these processes are what determines whether Lewy bodies form and cells die. One of the players may be the proteins that interact with α-synuclein; proteomics has offered us a powerful approach to identify all the proteins that are associated with α-synuclein in different experimental or disease conditions. Identification and characterization of these proteins will not only shed more light on the pathogenesis of PD, but will also yield potential therapeutic targets that can prevent PD development or halt its progression clinically.

References

1. Lowe, J., Graham, L., and Leigh, P.N. (1997) Disorders of movement and system degeneration. In *Greenfield's Neuropathology,* Lantos, P.L., ed. 1997, Arnold: London. pp. 281–366.
2. Li, Y.J., Scott, W.K., Hedges, D.J., Zhang, F., Gaskell, P.C., Nance, M.A., et al. (2002) Age at onset in two common neurodegenerative diseases is genetically controlled. *Am. J. Hum. Genet.* **70,** 985–993.
3. Maher, N.E., Golbe, L.I., Lazzarini, A.M., Mark, M.H., Currie, L.J., Wooten, G.F., et al. (2002) Epidemiologic study of 203 sibling pairs with Parkinson's disease: the GenePD study. *Neurology* **58,** 79–84.
4. Montgomery, E.B., Jr. (1995) Heavy metals and the etiology of Parkinson's disease and other movement disorders. *Toxicology* **97,** 3–9.
5. Langston, J.W. (1998) Epidemiology versus genetics in Parkinson's disease: progress in resolving an age-old debate. *Ann. Neurol.* **44,** S45–52.
6. Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., et al. (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* **276,** 2045–2047.
7. Leroy, E., Boyer, R., Auburger, G., Leube, B., Ulm, G., Mezey, E., et al. (1998) The ubiquitin pathway in Parkinson's disease. *Nature* **395,** 451–452.
8. Shimura, H., Schlossmacher, M.G., Hattori, N., Frosch, M.P., Trockenbacher, A., Schneider, R., et al. (2001) Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease. *Science* **293,** 263–269.
9. Alam, Z.I., Daniel, S.E., Lees, A.J., Marsden, D.J., Jenner, P., and Halliwell, B. (1997) A generalised increase in protein carbonyls in the brain in Parkinson's but not incidental Lewy body disease. *J. Neurochem.* **69,** 1326–1329.
10. Yoritaka, A., Hattori, N., Uchida, K., Tanaka, M., Stadtman, E.R., and Mizuno, Y. (1997) Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease. *Proc. Natl. Acad. Sci. USA* **93,** 2696–2701.
11. Lopiano, L., Fasano, M., Giraudo, S., Digilio, G., Koenig, S.H., Torre, E., et al. (2000) Nuclear magnetic relaxation dispersion profiles of substantia nigra pars compacta in Parkinson's disease patients are consistent with protein aggregation. *Neurochem. Int.* **37,** 331–336.
12. McNaught, K.S., Belizaire, R., Jenner, P., Olanow, C.W., and Isacson, O. (2002) Selective loss of 20S proteasome alpha-subunits in the substantia nigra pars compacta in Parkinson's disease. *Neurosci. Lett.* **326,** 155–158.
13. McNaught, K.S., Belizaire, R., Isacson, O., Jenner, P., and Olanow, C.W. (2003) Altered proteasomal function in sporadic Parkinson's disease. *Exp. Neurol.* **179,** 38–46.
14. McNaught, K.S. and Jenner, P. (2001) Proteasomal function is impaired in substantia nigra in Parkinson's disease. *Neurosci. Lett.* **297,** 191–194.
15. Maries, E., Dass, B., Collier, T.J., Kordower, J.H., and Steece-Collier, K. (2003) The role of alpha-synuclein in Parkinson's disease: insights from animal models. *Nat. Rev. Neurosci.* **4,** 727–738.
16. Warner, T.T. and Schapira, A.H. (2003) Genetic and environmental factors in the cause of Parkinson's disease. *Ann. Neurol.* **53,** S16–23; discussion S23–25.
17. Dawson, T.M. and Dawson, V.J. (2003) Rare genetic mutations shed light on the pathogenesis of Parkinson disease. *J. Clin. Invest.* **111,** 145–151.
18. Jenner, P. and Olanow, C.W. (1998) Understanding cell death in Parkinson's disease. *Ann. Neurol.* **44,** S72–84.
19. Polymeropoulos, M.H. (1998) Autosomal dominant Parkinson's disease. *J. Neurol.* **245,** P1–3.
20. Saha, A.R., Ninkina, N.N., Hanger, D.P., Anderton, B.H., Davies, A.M., and Buchman, V.L. (2000) Induction of neuronal death by alpha-synuclein. *Eur. J. Neurosci.* **12,** 3073–3077.
21. Trojanowski, J.Q. and Lee, L.M. (2000) "Fatal attractions" of proteins. A comprehensive

hypothetical mechanism underlying Alzheimer's disease and other neurodegenerative disorders. *Ann. NY Acad. Sci.* **924,** 62–67.
22 Lee, M.K., Stirling, W., Xu, Y., Xu, X., Qui, D., Mandir, A.S., et al. (2002) Human alpha-synuclein-harboring familial Parkinson's disease-linked Ala-53 → Thr mutation causes neurodegenerative disease with alpha-synuclein aggregation in transgenic mice. *Proc. Natl. Acad. Sci. USA* **99,** 8968–8973.
23 Jellinger, K.A. (2002) Recent developments in the pathology of Parkinson's disease. *J. Neural Transm. Suppl.* 347–376.
24 Sherer, T.B., Betarbet, R., Stout, A.K., Lund, S., Baptista, M., Panov, A.V., et al. (2002) An in vitro model of Parkinson's disease: linking mitochondrial impairment to altered alpha-synuclein metabolism and oxidative damage. *J. Neurosci.* **22,** 7006–7015.
25 Goldberg, M.S. and Lansbury, P.T., Jr. (2000) Is there a cause-and-effect relationship between alpha-synuclein fibrillization and Parkinson's disease? *Nat. Cell Biol.* **2,** E115–119.
26 Welch, W.J. and Gambetti, P. (1998) Chaperoning brain diseases. *Nature* **392,** 23–24.
27 Voges, D., Zwickl, P., and Baumeister, W. (1999) The 26S proteasome: a molecular machine designed for controlled proteolysis. *Annu. Rev. Biochem.* **68,** 1015–1068.
28 Johnston, J.A., Ward, C.L., and Kopito, R.R. (1998) Aggresomes: a cellular response to misfolded proteins. *J. Cell Biol.* **143,** 1883–1898.
29 Piccinini, M., Tazartes, O., Mostert, M., Musso, A., DeMarchi, M. and Rinaudo, M.T. (2000) Structural and functional characterization of 20S and 26S proteasomes from bovine brain. *Brain Res. Mol. Brain Res.* **76,** 103–114.
30. Liu, C.W., Millen, L., Roman, T.B., Xiong, H., Gilbert, H.F., Noiva, R., et al. (2002) Conformational remodeling of proteasomal substrates by PA700, the 19 S regulatory complex of the 26 S proteasome. *J. Biol. Chem.* **277,** 26,815–26,820.
31 DeMartino, G.N. and Slaughter, C.A. (1999) The proteasome, a novel protease regulated by multiple mechanisms. *J. Biol. Chem.* **274,** 22,123–22,126.
32 Pickart, C.M. and VanDemark, A.P. (2000) Opening doors into the proteasome. *Nat. Struct. Biol.* **7,** 999–1001.
33 Shringarpure, R., Grune, T., Mehlhase, J., and Davies, K.J. (2002) Ubiquitin-conjugation is not required for the degradation of oxidized proteins by the proteasome. *J. Biol. Chem.* **278,** 311–318.
34 Tu, P.H., Galvin, J.E., Baba, M., Giasson, B., Tomita, T., Leight, S., et al. (1998) Glial cytoplasmic inclusions in white matter oligodendrocytes of multiple system atrophy brains contain insoluble alpha-synuclein. *Ann. Neurol.* **44,** 415–422.
35 Galvin, J.E., Giasson, B., Hurtig,, H.I., Lee, V.M. and Trojanowski, J.Q. (2000) Neurodegeneration with brain iron accumulation, type 1 is characterized by alpha-, beta-, and gamma-synuclein neuropathology. *Am. J. Pathol.* **157,** 361–368.
36 Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., et al. (1998) Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nat. Genet.* **18,** 106–108.
37 Tofaris, G.K., Layfield, R., and Spillantini, M.G. (2001) Alpha-synuclein metabolism and aggregation is linked to ubiquitin-independent degradation by the proteasome. *FEBS Lett.* **509,** 22–26.
38 Tanaka, Y., Engelender, S., Igarashi, S., Rao, R.K., Wanner, T., Tanzi, R.E., et al. (2001) Inducible expression of mutant alpha-synuclein decreases proteasome activity and increases sensitivity to mitochondria-dependent apoptosis. *Hum. Mol. Genet.* **10,** 919–926.
39 Hsu, L.J., Sagara, Y., Arroyo, A., Rockenstein, E., Sisk, A., Mallory, M., et al. (2000) Alpha-synuclein promotes mitochondrial deficit and oxidative stress. *Am. J. Pathol.* **157,** 401–410.
40 Elkon, H., Don, J., Melamed, E., Ziv, I., Shirvan, A., and Offen, D. (2002) Mutant and wild-type alpha-synuclein interact with mitochondrial cytochrome C oxidase. *J. Mol. Neurosci.* **18,** 229–238.
41 Abeliovich, A., Schmitz, Y., Farinas, I., Choi-Lundberg, D., Ho, W.H., Castillo, P.E., et al. (2000) Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. *Neuron* **25,** 239–252.
42 Dauer, W., Kholodilov, N., Vila, M., Trillat, A.C., Goodchild, R., Larsen, K.E., et al. (2002) Resistance of alpha-synuclein null mice to the parkinsonian neurotoxin MPTP. *Proc. Natl. Acad. Sci. USA* **99,** 14,524–14,529.
43 Masliah, E., Rockenstein, E., Veinbergs, I., Mallory, M., Hashimoto, M., Takeda, A., et al. (2000) Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. *Science* **287,** 1265–1269.

44 Feany, M.B. and Bender, W.W. (2000) A *Drosophila* model of Parkinson's disease. *Nature* **404,** 394–398.
45 Kirik, D., Rosenblad, C., Burger, C., Lundberg, C., Johansen, T.E., Muzyczka, N., et al. (2002) Parkinson-like neurodegeneration induced by targeted overexpression of alpha-synuclein in the nigrostriatal system. *J. Neurosci.* **22,** 2780–2791.
46 Auluck, P.K., Chan, H.Y., Trojanowski, J.Q., Lee, V.M., and Bonini, N.M. (2002) Chaperone suppression of alpha-synuclein toxicity in a *Drosophila* model for Parkinson's disease. *Science* **295,** 865–868.
47 Singleton, A.B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kachergus, J., et al. (2003) Alpha-synuclein locus triplication causes Parkinson's disease. *Science* **302,** 841.
48 Schlossmacher, M.G., Frosch, M.P., Gai, W.P., Medina, M., Sharma, N., Forno, L., et al. (2002) Parkin localizes to the Lewy bodies of Parkinson disease and dementia with Lewy bodies. *Am. J. Pathol.* **160,** 1655–1667.
49 McNaught, K.S., Olanow, C.W., Halliwell, B., Isacson, O., and Jenner, P. (2001) Failure of the ubiquitin-proteasome system in Parkinson's disease. *Nat. Rev. Neurosci.* **2,** 589–594.
50 McNaught, K.S., Bjorklund, L.M., Belizaire, R., Isacson, O., Jenner, P., and Olanow, C.W. (2002) Proteasome inhibition causes nigral degeneration with inclusion bodies in rats. *Neuroreport* **13,** 1437–1441.
51 Nam, S., Smith, D.M., and Dou, Q.P. (2001) Ester bond-containing tea polyphenols potently inhibit proteasome activity in vitro and in vivo. *J. Biol. Chem.* **276,** 13,322–13,330.
52. Bulteau, A.L., Lundberg, K.C., Humphries, K.M., Sadek, H.A., Szweda, P.A., Friguet, B., et al. (2001) Oxidative modification and inactivation of the proteasome during coronary occlusion/reperfusion. *J. Biol. Chem.* **276,** 30,057–30,063.
53 Fink, A.L. (1999) Chaperone-mediated protein folding. *Physiol. Rev.* **79,** 425–449.
54. Zhou, H., Li, S.H., and Li, X.J. (2001) Chaperone suppression of cellular toxicity of huntingtin is independent of polyglutamine aggregation. *J. Biol. Chem.* **276,** 48,417–48,424.
55 Auluck, P.K. and Bonini, N.M. (2002) Pharmacological prevention of Parkinson disease in *Drosophila*. *Nat. Med.* **8,** 1185–1186.
56 Sharon, R., Bar-Joseph, I., Frosch, M.P., Walsh, D.M., Hamilton, J.M., and Selkoe, D.J. (2003) The formation of highly soluble oligomers of alpha-synuclein is regulated by fatty acids and enhanced in Parkinson's disease. *Neuron* **37,** 583–595.
57 Volles, M.J., Lee, S.J., Rochet, J.C., Shtilerman, M.D., Ding, T.T., Kessler, J.C., et al (2001) Vesicle permeabilization by protofibrillar alpha-synuclein: implications for the pathogenesis and treatment of Parkinson's disease. *Biochemistry* **40,** 7812–7819.
58 Gosavi, N., Lee, H.J., Lee, J.S., Patel, S., and Lee, S.J. (2002) Golgi fragmentation occurs in the cells with prefibrillar alpha-synuclein aggregates and precedes the formation of fibrillar inclusion. *J. Biol. Chem.* **277,** 48,984–48,992.
59 Bucciantini, M., Giannoni, E., Chiti, F., Baroni, F., Formigli, L., Zurdo, J., et al. (2002) Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature* **416,** 507–511.
60 Ding, T.T., Lee, S.J., Rochet, J.C., and Lansbury, P.T., Jr. (2002) Annular alpha-synuclein protofibrils are produced when spherical protofibrils are incubated in solution or bound to brain-derived membranes. *Biochemistry* **41,** 10,209–10,217.
61 Neumann, M., Kahle, P.J., Giasson, B.I., Ozmen, L., Borroni, E., Spooren, W., et al. (2002) Misfolded proteinase K-resistant hyperphosphorylated alpha-synuclein in aged transgenic mice with locomotor deterioration and in human alpha-synucleinopathies. *J. Clin. Invest.* **110,** 1429–1439.
62 Dev, K.K., Hofele, K., Barbieri, S., Buchman, V.L., and van der Putten, H. (2003) Part II: alpha-synuclein and its molecular pathophysiological role in neurodegenerative disease. *Neuropharmacology* **45,** 14–44.
63 Zhang, J., Perry, G., Smith, M.A., Robertson, D., Olson, S.J., Graham, D.G. et al. (1999) Parkinson's disease is associated with oxidative damage to cytoplasmic DNA and RNA in substantia nigra neurons. *Am. J. Pathol.* **154,** 1423–1429.
64 Giasson, B.I., Ischiropoulos, H., Lee, V.M., and Trojanowski, J.Q. (2002) The relationship between oxidative/nitrative stress and pathological inclusions in Alzheimer's and Parkinson's diseases. *Free Radic. Biol. Med.* **32,** 1264–1275.
65 Wallace, D.C. (1992) Mitochondrial genetics: a paradigm for aging and degenerative diseases? *Science* **256,** 628–632.

66 Chance, B. (1973) The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem. J.* **134,** 707–716.
67 Simon, D.K., Mayeux, R., Marder, K., Kowall, N.W., Beal, M.F., and Johns, D.R. (2000) Mitochondrial DNA mutations in complex I and tRNA genes in Parkinson's disease. *Neurology* **54,** 703–709.
68 Haas, R.H., Nasirian, F., Nakano, K., Ward, D., Pay, M., Hill, R., et al. (1995) Low platelet mitochondrial complex I and complex II/III activity in early untreated Parkinson's disease. *Ann. Neurol.* **37,** 714–722.
69 Benecke, R., Strumper, P., and Weiss, H. (1993) Electron transfer complexes I and IV of platelets are abnormal in Parkinson's disease but normal in Parkinson-plus syndromes. *Brain* **116,** 1451–1463.
70 Schapira, A.H., Cooper, J.M., Dexter, D., Jenner, P., Clark, J.B., and Marsden, C.D. (1989) Mitochondrial complex I deficiency in Parkinson's disease. *Lancet* **1,** 1269.
71 Shults, C.W., Haas, R.H., and Beal, M.F. (1999) A possible role of coenzyme Q10 in the etiology and treatment of Parkinson's disease. *Biofactors* **9,** 267–272.
72 Coenen, M.J., van den Heuvel, L.P., and Smeitink, J.A. (2001) Mitochondrial oxidative phosphorylation system assembly in man: recent achievements. *Curr. Opin. Neurol.* **14,** 777–781.
73 Jansen, R.P. (2000) Origin and persistence of the mitochondrial genome. *Hum. Reprod.* **15,** 1–10.
74 Swerdlow, R.H., Parks, J.K., Miller, S.W., Tuttle, J.B., Trimmer, P.A., Sheehan, J.P. et al. (1996) Origin and functional consequences of the complex I defect in Parkinson's disease. *Ann. Neurol.* **40,** 663–671.
75 Mizuno, Y., Suzuki, K., and Ohta, S. (1990) Postmortem changes in mitochondrial respiratory enzymes in brain and a preliminary observation in Parkinson's disease. *J. Neurol. Sci.* **96,** 49–57.
76 Zhang, J., Montine, T.J., Smith, M.A., Siedlak, S.L., Gu, G., Robertson, D., et al. (2002) The mitochondrial common deletion in Parkinson's disease and related movement disorders. *Parkinsonism & Relat. Disord.* **8,** 165–170.
77 Gu, G., Reyes, P.E., Golden, G.T., Woltjer, R.L, Hulette, C., Montine, T.J., et al. (2002) Mitochondrial DNA deletions/rearrangements in parkinson disease and related neurodegenerative disorders. *J. Neuropathol. Exp. Neurol.* **61,** 634–639.
78 Patterson, S.D., Spahr, C.S., Daugas, E., Susin, S.A., Irinopoulou, T., Koehler, C., et al. (2000) Mass spectrometric identification of proteins released from mitochondria undergoing permeability transition. *Cell Death Differ.* **7,** 137–144.
79 Lopez, M.F. and Melov, S. (2002) Applied proteomics: mitochondrial proteins and effect on function. *Circ. Res.* **90,** 380–389.
80 Droge, W. (2002) Free radicals in the physiological control of cell function. *Physiol. Rev.* **82,** 47–95.
81 Liu, X., Kim, C.N., Yang, J., Jemmerson, R., and Wang, X. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* **86,** 147–157.
82 Beal, M.F. (1992) Does impairment of energy metabolism result in excitotoxic neuronal death in neurodegenerative illnesses? *Ann. Neurol.* **31,** 119–130.
83 Beal, M.F. (1995) Aging, energy, and oxidative stress in neurodegenerative diseases. *Ann. Neurol.* **38,** 357–366.
84 Hoyt, K.R., Reynolds, I.J., and Hastings, T.G. (1997) Mechanisms of dopamine-induced cell death in cultured rat forebrain neurons: interactions with and differences from glutamate-induced cell death. *Exp. Neurol.* **143,** 269–281.
85 Sriram, K., Pai, K.S., Boyd, M.R., and Ravindranath, V. (1997) Evidence for generation of oxidative stress in brain by MPTP: in vitro and in vivo studies in mice. *Brain Res.* **749,** 44–52.
86 Zhang, J. and Piantadosi, C.A. (1992) Mitochondrial oxidative stress after carbon monoxide hypoxia in the rat brain. *J. Clin. Invest.* **90,** 1193–1199.
87 Cohen, G. (1983) The pathobiology of Parkinson's disease: biochemical aspects of dopamine neuron senescence. *J. Neural Transm. Suppl.* **19,** 89–103.
88 Graham, D.G. (1978) Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones. *Mol. Pharmacol.* **14,** 633–643.
89 Graham, D.G., Tiffany, S.M., Bell, W.R., Jr., and Gutknecht, W.F. (1978) Autoxidation versus covalent binding of quinones as the mechanism of toxicity of dopamine, 6-hydroxydopamine, and related compounds toward C1300 neuroblastoma cells in vitro. *Mol. Pharmacol.* **14,** 644–653.

90 Li, H. and Dryhurst, G. (1997) Irreversible inhibition of mitochondrial complex I by 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2H-1,4-benzothiazine-3-carboxylic acid (DHBT-1): a putative nigral endotoxin of relevance to Parkinson's disease. *J. Neurochem.* **69,** 1530–1541.

91 Montine, T.J., Picklo, M.J., Amarnath, V., Whetsell, W.O., Jr., and Graham, D.G. (1997) Neurotoxicity of endogenous cysteinylcatechols. *Exp. Neurol.* **148,** 26–33.

92 Zhang, J., Kravtsov, V., Amarnath, V., Picklo, M.J., Graham, D.G., and Montine, T.J. (2000) Enhancement of dopaminergic neurotoxicity by the mercapturate of dopamine: relevance to Parkinson's disease. *J. Neurochem.* **74,** 970–978.

93 Li, C.L., Werner, P., and Cohen, G. (1995) Lipid peroxidation in brain: interactions of L-DOPA/dopamine with ascorbate and iron [published erratum appears in *Neurodegeneration* 1995 Sep;4(3):347]. *Neurodegeneration* **4,** 147–153.

94 Shringarpure, R., Grune, T., and Davies, K.J. (2001) Protein oxidation and 20S proteasome-dependent proteolysis in mammalian cells. *Cell Mol. Life Sci.* **58,** 1442–1450.

95 Munch, G., Luth, H.J., Wong, A., Arendt, T., Hirsch, E., Ravid, R., et al. (2000) Crosslinking of alpha-synuclein by advanced glycation end-products—an early pathophysiological step in Lewy body formation? *J. Chem. Neuroanat.* **20,** 253–257.

96 Giasson, B.I., Duda, J.E., Murray, I.V., Chen, Q., Souza, J.M., Hurtig, H.I., et al. (2000) Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. *Science* **290,** 985–989.

97. Souza, J.M., Giasson, B.I., Chen, Q., Lee, V.M., and Ischiropoulos, H. (2000) Dityrosine cross-linking promotes formation of stable alpha-synuclein polymers. Implication of nitrative and oxidative stress in the pathogenesis of neurodegenerative synucleinopathies. *J. Biol. Chem.* **275,** 18,344–18,349.

98 Davies, K.J. (2001) Degradation of oxidized proteins by the 20S proteasome. *Biochimie* **83,** 301–310.

99 Sulzer, D. (2001) Alpha-synuclein and cytosolic dopamine: stabilizing a bad situation. *Nat. Med.* **7,** 1280–1282.

100 Conway, K.A., Rochet, J.C., Bieganski, R.M., and Lansbury, P.T., Jr. (2001) Kinetic stabilization of the alpha-synuclein protofibril by a dopamine-alpha-synuclein adduct. *Science* **294,** 1346–1349.

101 Rinaldi, T., Ricci, C., Porro, D., Bolotin-Fukuhara, M., and Frontali, L. (1998) A mutation in a novel yeast proteasomal gene, RPN11/MPR1, produces a cell cycle arrest, overreplication of nuclear and mitochondrial DNA, and an altered mitochondrial morphology. *Mol. Biol. Cell* **9,** 2917–2931.

102 Campbell, C.L., Tanaka, N., White, K.H., and Thorsness, P.E. (1994) Mitochondrial morphological and functional defects in yeast caused by yme1 are suppressed by mutation of a 26S protease subunit homologue. *Mol. Biol. Cell* **5,** 899–905.

103 Marshansky, V., Wang, X., Bertrand, R., Luo, H., Duguid, W., Chinnadurai, G., et al. (2001) Proteasomes modulate balance among proapoptotic and antiapoptotic Bcl-2 family members and compromise functioning of the electron transport chain in leukemic cells. *J. Immunol.* **166,** 3130–3142.

104 MacFarlane, M., Merrison, W., Bratton, S.B., and Cohen, G.M. (2002) Proteasome-mediated degradation of Smac during apoptosis: XIAP promotes Smac ubiquitination in vitro. *J. Biol. Chem.* **277,** 36,611–36,616.

105 Trojanowski, J.Q. and Lee, V.M. (2002) Parkinson's disease and related synucleinopathies are a new class of nervous system amyloidoses. *Neurotoxicology* **23,** 457–460.

106 Masliah, E. and Hashimoto, M. (2002) Development of new treatments for Parkinson's disease in transgenic animal models: a role for beta-synuclein. *Neurotoxicology* **23,** 461–468.

107 Kirik, D., Annett, L.E., Burger, C., Muzyczka, N., Mandel, R.J., and Bjorklund, A. (2003) Nigrostriatal alpha-synucleinopathy induced by viral vector-mediated overexpression of human alpha-synuclein: a new primate model of Parkinson's disease. *Proc. Natl. Acad. Sci. USA* **100,** 2884–2889.

108 Betarbet, R., Sherer, T.B., MacKenzie, G., Garcia-Osuna, M., Panov, A.V., and Greenamyre, T.J. (2000) Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat. Neurosci.* **3,** 1301–1306.

109 Sherer, T.B., Kim, J.H., Betarbet, R., and Greenamyre, J.T. (2003) Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and alpha-synuclein aggregation. *Exp. Neurol.* **179,** 9–16.

110 Lee, H.J., Shin, S.Y., Choi, C., Lee, Y.H., and Lee, S.J. (2002) Formation and removal of alpha-synuclein aggregates in cells exposed to

mitochondrial inhibitors. *J. Biol. Chem.* **277,** 5411–5417.
111 Seo, B.B., Nakamaru-Ogiso, E., Flotte, T.R., Yagi, T., and Matsuno-Yagi, A. (2002) A single-subunit NADH-quinone oxidoreductase renders resistance to mammalian nerve cells against complex I inhibition. *Mol. Ther.* **6,** 336–341.
112 Chiueh, C.C., Wu, R.M., Mohanakumar, K.P., Sternberger, L.M., Krishna, G., Obata, T., et al. (1994) In vivo generation of hydroxyl radicals and MPTP-induced dopaminergic toxicity in the basal ganglia. *Ann. NY Acad. Sci.* **738,** 25–36.
113 Di Monte, D.A., Royland, J.E., Anderson, A., Castagnoli, K., Castagnoli, N., Jr., and Langston, J.W. (1997) Inhibition of monoamine oxidase contributes to the protective effect of 7-nitroindazole against MPTP neurotoxicity. *J. Neurochem.* **69,** 1771–1773.
114 Langston, J.W., Ballard, P., Tetrud, J.W., and Irwin, I. (1983) Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* **219,** 979–980.
115. Gerlach, M. and Riederer, P. (1996) Animal models of Parkinson's disease: an empirical comparison with the phenomenology of the disease in man. *J. Neural Trans.* **103,** 987–1041.
116 Heikkila, R.E., Nicklas, W.J., Vyas, I., and Duvoisin, R.C. (1985) Dopaminergic toxicity of rotenone and the 1-methyl-4-phenylpyridinium ion after their stereotaxic administration to rats: implication for the mechanism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity. *Neurosci. Lett.* **62,** 389–394.
117. Forno, L.S., DeLanney, L.E., Irwin, I., and Langston, J.W. (1993) Similarities and differences between MPTP-induced parkinsonsim and Parkinson's disease. Neuropathologic considerations. *Adv. Neurol.* **60,** 600–608.
118 Elsworth, J.D., Taylor, J.R., Sladek, J.R., Jr., Collier, T.J., Redmond, D.E., Jr., and Roth, R.H. (2000) Striatal dopaminergic correlates of stable parkinsonism and degree of recovery in old-world primates one year after MPTP treatment. *Neuroscience* **95,** 399–408.
119 Collier, T.J., Sortwell, C.E., Elsworth, J.D., Taylor, J.R. Roth, R.H., Sladek, J.R. Jr., et al. (2002) Embryonic ventral mesencephalic grafts to the substantia nigra of MPTP-treated monkeys: feasibility relevant to multiple-target grafting as a therapy for Parkinson's disease. *J. Comp. Neurol.* **442,** 320–330.
120 Gomez-Santos, C., Ferrer, I., Reiriz, J., Vinals, F., Barrachina, M., and Ambrosio, S. (2002) MPP+ increases alpha-synuclein expression and ERK/MAP-kinase phosphorylation in human neuroblastoma SH-SY5Y cells. *Brain Res.* **935,** 32–39.
121 Vila, M., Vukosavic, S., Jackson-Lewis, V., Neystat, M., Jakowec, M., and Przedborski, S. (2000) Alpha-synuclein up-regulation in substantia nigra dopaminergic neurons following administration of the parkinsonian toxin MPTP. *J. Neurochem.* **74,** 721–729.
122 Przedborski, S., Chen, Q., Vila, M., Giasson, B.I., Djaldatti, R., Vukosavic, S., et al. (2001) Oxidative post-translational modifications of alpha-synuclein in the 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP) mouse model of Parkinson's disease. *J. Neurochem.* **76,** 637–640.
123 Meredith, G.E., Totterdell, S., Petroske, E., Santa Cruz, K., Callison, R.C., and Lau, Y.S. (2002) Lysosomal malfunction accompanies alpha-synuclein aggregation in a progressive mouse model of Parkinson's disease. *Brain Res.* **956,** 156–165.
124 Kowall, N.W., Hantraye, P., Brouillet, E., Beal, M.F., McKee, A.C., and Ferrante, R.J. (2000) MPTP induces alpha-synuclein aggregation in the substantia nigra of baboons. *Neuroreport* **11,** 211–213.
125 Lehmensiek, V., Tan, E.M., Schwarz, J., and Storch, A. (2002) Expression of mutant alpha-synucleins enhances dopamine transporter-mediated MPP+ toxicity in vitro. *Neuroreport* **13,** 1279–1283.
126 Hasegawa, E., Takeshige, K., Oishi, T., Murai, Y., and Minakami, S. (1990) 1-Methyl-4-phenylpyridinium (MPP+) induces NADH-dependent superoxide formation and enhances NADH-dependent lipid peroxidation in bovine heart submitochondrial particles. *Biochem. Biophys. Res. Comm.* **170,** 1049–1055.
127 Bezard, E., Gross, C.E., Fournier, M.C., Dovero, S., Bloch, B., and Jaber, M. (1999) Absence of MPTP-induced neuronal death in mice lacking the dopamine transporter. *Exp. Neurol.* **155,** 268–273.
128 Zuddas, A., Fascetti, F., Corsini, G.U., and Piccardi, M.P. (1994) In brown Norway rats, MPP+ is accumulated in the nigrostriatal dopaminergic terminals but it is not neurotoxic: a model of natural resistance to MPTP toxicity. *Exp. Neurol.* **127,** 54–61.

129 Altar, C.A., Heikkila, R.E., Manzino, L., and Marien, M.R. (1986) 1-Methyl-4-phenylpyridine (MPP+): regional dopamine neuron uptake, toxicity, and novel rotational behavior following dopamine receptor proliferation. *Eur. J. Pharmacol.* **131,** 199–209.

130. Obata, T. and Chiueh, C.C. (1992) In vivo trapping of hydroxyl free radicals in the striatum utilizing intracranial microdialysis perfusion of salicylate: effects of MPTP, MPDP+, and MPP+. *J. Neural Transm. Gen. Sect.* **89,** 139–145.

131 Espino, A., Llorens, J., Calopa, M., Bartrons, R., Rodriguez-Farre, E., and Ambrosio, S. (1995) Cerebrospinal dopamine metabolites in rats after intrastriatal administration of 6-hydroxydopamine or 1-methyl-4-phenylpyridinium ion. *Brain Res.* **669,** 19–25.

132 Ghorayeb, I., Fernagut, P.O., Hervier, L., Labattu, B., Bioulac, B., and Tison, F. (2002) A "single toxin-double lesion" rat model of striatonigral degeneration by intrastriatal 1-methyl-4-phenylpyridinium ion injection: a motor behavioural analysis. *Neuroscience* **115,** 533–546.

133 Okada, M., Yokotani, K., Yamashita, M., Ikeda, H., and Osumi, Y. (1989) Central depletion of dopamine in rats by 1-methyl-4-phenylpyridine. *Life Sci.* **45,** 391–399.

134 Steffen, V., Santiago, M., de la Cruz, C.P., Revilla, E., Machado, A., and Cano, J. (1995) Effect of intraventricular injection of 1-methyl-4-phenylpyridinium: protection by acetyl-L-carnitine. *Hum. Exp. Toxicol.* **14,** 865–871.

135 Zigmond, M.J., Hastings, T.J., and Abercrombie, E.D. (1992) Neurochemical responses to 6-hydroxydopamine and L-dopa therapy: implications for Parkinson's disease. *Ann. NY Acad. Sci.* **648,** 71–86.

136. Ungerstedt, U. (1971) Postsynaptic supersensitivity after 6-hydroxydopamine induced degeneration of the nigrostriatal dopamine system. *Acta Physiol. Scand. Suppl.* **367,** 51–68.

137 Guan, J., Krishnamurthi, R., Waldvogel, H.L., Faull, R.L., Clark, R., and Gluckman, P. (2000) N-terminal tripeptide of IGF-1 (GPE) prevents the loss of TH positive neurons after 6-OHDA induced nigral lesion in rats. *Brain Res.* **859,** 286–292.

138. Bergdall, V.K. and Becker, J.B. (1994) Effects of nerve growth factor infusion on behavioral recovery and graft survival following intraventricular adrenal medulla grafts in the unilateral 6-hydroxydopamine lesioned rat. *J. Neural Transplant Plast.* **5,** 163–167.

139 Harik, S.I., LaManna, J.C., Snyder, S., Wetherbee, J.R., and Rosenthal, M. (1982) Abnormalities of cerebral oxidative metabolism in animal models of Parkinson disease. *Neurology* **32,** 382–389.

140 Mandel, R.J. (2000) Effect of acute L-dopa pretreatment on apomorphine-induced rotational behavior in a rat model of Parkinson's disease. *Exp. Neurol.* **161,** 212–219.

141 Carrasco, E. and Werner, P. (2002) Selective destruction of dopaminergic neurons by low concentrations of 6-OHDA and MPP(+): protection by acetylsalicylic acid aspirin. *Parkinsonism Relat. Disord.* **8,** 407–411.

142 Hefti, F., Melamed, E., Sahakian, B.J., and Wurtman, R.J. (1980) Circling behavior in rats with partial, unilateral nigro-striatal lesions: effect of amphetamine, apomorphine, and DOPA. *Pharmacol. Biochem. Behav.* **12,** 185–188.

143 Heikkila, R.E. and Cohen, G. (1972) In vivo generation of hydrogen peroxide from 6-hydroxydopamine. *Experiential* **28,** 1197–1198.

144 Saner, A. and Thoenen, H. (1971) Model experiments on the molecular mechanism of action of 6-hydroxydopamine. *Mol. Pharmacol.* **7,** 147–154.

145. Cohen, G. (1987) Monoamine oxidase, hydrogen peroxide, and Parkinson's disease. *Adv. Neurol.* **45,** 119–125.

146 Park, J.W., Youn, Y.C., Kwon, O.S., Jang, Y.Y., Han, E.S., and Lee, C.S. (2002) Protective effect of serotonin on 6-hydroxydopamine- and dopamine-induced oxidative damage of brain mitochondria and synaptosomes and PC12 cells. *Neurochem. Int.* **40,** 223–233.

147 Dabbeni-Sala, F., Di Santo, S., Franceschini, D., Skaper, S.D., and Giusti, P. (2001) Melatonin protects against 6-OHDA-induced neurotoxicity in rats: a role for mitochondrial complex I activity. *FASEB J.* **15,** 164–170.

148 Glinka, Y., Tipton, K.F., and Youdim, M.B. (1998) Mechanism of inhibition of mitochondrial respiratory complex I by 6-hydroxydopamine and its prevention by desferrioxamine. *Eur. J. Pharmacol.* **351,** 121–129.

149 Curtius, H.C., Wolfensberger, M., Steinmann, B., Redweik, U., and Siegfried, J. (1974) Mass fragmentography of dopamine and 6-hydroxydopamine. Application to the determination of dopamine in human brain biopsies from the caudate nucleus. *J. Chromatogr.* **99,** 529–540.

150 Blum, D., Torch, S., Lambeng, N., Nissou, M., Benabid, A.L., Sadoul, R., et al. (2001) Molecu-

lar pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. *Prog. Neurobiol.* **65,** 135–172.
151 Elkon, H., Melamed, E., and Offen, D. (2001) 6-Hydroxydopamine increases ubiquitin-conjugates and protein degradation: implications for the pathogenesis of Parkinson's disease. *Cell Mol. Neurobiol.* **21,** 771–781.
152 Langston, J.W., Forno, L.S., Rebert, C.S., and Irwin, I. (1984) Selective nigral toxicity after systemic administration of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyrine (MPTP) in the squirrel monkey. *Brain Res.* **292,** 390–394.
153 McKeith, I.G., Galasko, D., Kosaka, K., Perry, E.R., Dickson, D.W., Hansen, L.A., et al. (1996) Consensus guidelines for the clinical and pathologic diagnosis of dementia with Lewy bodies (DLB): report of the consortium on DLB international workshop. *Neurology* **47,** 1113–1124.
154 Chait, B.T. and Kent, S.B. (1992) Weighing naked proteins: practical, high-accuracy mass measurement of peptides and proteins. *Science* **257,** 1885–1894.
155 Benvenuti, S., Cramer, R., Bruce, J., Waterfield, M.D., and Jat, P.S. (2002) Identification of novel candidates for replicative senescence by functional proteomics. *Oncogene* **21,** 4403–4413.
156 Castegna, A., Aksenov, M., Thongboonkerd, V., Klein, J.B., Pierce, M.B., Booze, R., et al. (2002) Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part II: dihydropyrimidinase-related protein 2, alpha-enolase and heat shock cognate 71. *J. Neurochem.* **82,** 1524–1532.
157 Gygi, S.P., Corthals, G.L., Zhang, Y., Rochon, Y., and Aebersold, R. (2000) Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. *Proc. Natl. Acad. Sci. USA* **97,** 9390–9395.
158 Haynes, P.A. and Yates, J.R., 3rd. (2000) Proteome profiling-pitfalls and progress. *Yeast* **17,** 81–87.
159 Gygi, S.P., Rist, B., Gerber, S.A., Turecek, F., Gelb, M.H., and Aebersold, R. (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* **17,** 994–999.
160 Aebersold, R. and Goodlett, D.R. (2001) Mass spectrometry in proteomics. *Chem. Rev.* **101,** 269–295.
161 Link, A.J., Eng, J., Schieltz, D.M., Carmack, E., Mize, G.J., Morris, D.R., et al. (1999) Direct analysis of protein complexes using mass spectrometry. *Nat. Biotechnol.* **17,** 676–682.
162 Goodlett, D.R., Keller, A., Watts, J.D., Newitt, R., Yi, E.C., Purvine, S., et al. (2001) Differential stable isotope labeling of peptides for quantitation and de novo sequence derivation. *Rapid Commun. Mass Spectrom.* **15,** 1214–1221.
163 Goodlett, D.R. and Yi, E.C. (2002) Proteomics without polyacrylamide: qualitative and quantitative uses of tandem mass spectrometry in proteome analysis. *Funct. Integr. Genomics* **2,** 138–153.
164 Rappsilber, J., Ryder, U., Lamond, A.I., and Mann, M. (2002) Large-scale proteomic analysis of the human spliceosome. *Genome Res.* **12,** 1231–1245.
165 Rout, M.P., Aitchison, J.D., Suprapto, A., Hjertaas, K., Zhao, Y., and Chait, B.T. (2000) The yeast nuclear pore complex: composition, architecture, and transport mechanism. *J. Cell Biol.* **148,** 635–651.
166 Fountoulakis, M., Berndt, P., Langen, H., and Suter, L. (2002) The rat liver mitochondrial proteins. *Electrophoresis* **23,** 311–328.
167 Bergquist, J., Gobom, J., Blomberg, A., Roepstorff, P., and Ekman, R. (2001) Identification of nuclei associated proteins by 2D-gel electrophoresis and mass spectrometry. *J. Neurosci. Methods* **109,** 3–11.
168 Oda, Y., Nagasu, T., and Chait, B.T. (2001) Enrichment analysis of phosphorylated proteins as a tool for probing the phosphoproteome. *Nat. Biotechnol.* **19,** 379–382.
169 Spahr, C.S., Susin, S.A., Bures, E.J., Robinson, J.H., Davis, M.T., McGinley, M.D., et al. (2000) Simplification of complex peptide mixtures for proteomic analysis: reversible biotinylation of cysteinyl peptides. *Electrophoresis* **21,** 1635–1650.
170 Haystead, C.M., Gregory, P., Sturgill, T.W., and Haystead, T.A. (1993) Gamma-phosphate-linked ATP-sepharose for the affinity purification of protein kinases. Rapid purification to homogeneity of skeletal muscle mitogen-activated protein kinase kinase. *Eur. J. Biochem.* **214,** 459–467.
171. Adam, G.C., Sorensen, E.J., and Cravatt, B.F. (2002) Proteomic profiling of mechanistically distinct enzyme classes using a common chemotype. *Nat. Biotechnol.* **20,** 805–809.