Ubiquitinated inclusions and neuronal cell death

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Summary. Ubiquitinated inclusions and selective neuronal cell death are considered the pathological hallmarks of Parkinson’s disease and other neurodegenerative diseases. Recent genetic, pathological and biochemical evidence suggests that dysfunction of ubiquitin-dependent protein degradation by the proteasome might be a contributing, if not initiating factor in the pathogenesis of these diseases. In neuronal cell culture models inhibition of the proteasome leads to cell death and formation of fibrillar ubiquitin and α-synuclein-positive inclusions, thus modeling some aspects of Lewy body diseases. The processes of inclusion formation and neuronal cell death share some common mechanisms, but can also be dissociated at a certain level.

Key words: Neurodegenerative, Apoptosis, Proteasome, Lewy body, Parkinson’s disease

Introduction

In this review, we first discuss some general concepts of ubiquitinated inclusion formation and neuronal death, as they pertain to neurodegenerative conditions, and in particular to Parkinson’s disease (PD) and other Lewy body (LB) diseases. We then address our own and others’ recent findings, which suggest that dysfunction of the ubiquitin-dependent degradation system may be involved in the pathogenesis of such disorders. Lastly, we describe our attempts to decipher molecular pathways involved in ubiquitinated inclusion formation and neuronal death.

Intracellular ubiquitinated inclusions and cell death in Parkinson’s disease and other neurodegenerative conditions

The prevalence of PD is 160/100,000 individuals, and it increases with age (Fahn, 1995). The pathological hallmark of PD is the combination of catecholaminergic neuron cell death and the presence of LBs in brainstem nuclei, primarily in the dopaminergic neurons of substantia nigra pars compacta (SNpc) (Gibb, 1993). LBs are filamentous cytoplasmic inclusions that stain with antibodies targeted against ubiquitin and α-synuclein (Gibb, 1993; Spillantini et al., 1997, 1998). It is increasingly being recognized that, in addition to brainstem, cortical Lewy bodies (CLBs) are often present in PD and in the related condition diffuse Lewy body disease (DLBD) (Papka et al., 1998; Cordato et al., 2000; Hurtig et al., 2000; Mattila et al., 2000). Oftentimes, Alzheimer’s disease (AD) and DLBD coexist, and the term LB variant of AD has been suggested (Hansen, 1997). Recent studies suggest that more than 50% of cases with sporadic AD and many with genetically determined AD manifest LBs, particularly in the amygdala (Lippa et al., 1998; Hamilton, 2000). It is apparent therefore that the formation of LBs is a major feature of a variety of neurodegenerative conditions that range from PD to AD. Their presence may provide insights into the underlying pathophysiology, regardless of whether these inclusions are pathogenic, protective, or just markers of the disease process. In other words, it is reasonable to assume that the same initiating factors lead to neuronal degeneration and to LB formation and that attempts to decipher the manner in which LBs are formed may lead to a better understanding of the disease process.

The ubiquitin-dependent proteolytic system

A major component of LBs is ubiquitin, a small protein of 76 amino acids. It is translated as proubiquitin, a string of multiple polypeptides, or as a fusion with ribosomal proteins, which are cleaved to monomeric ubiquitin molecules. Multiple molecules of ubiquitin attach as polyubiquitin chains to proteins, which are then signaled for degradation. The process of ubiquitination involves first the ATP-dependent activation of ubiquitin by a universal E1 ubiquitin-activating enzyme, then the transfer, by an E2 ubiquitin-
conjugating enzyme (UCE), of activated ubiquitin from E1 to a member of the E3 ligase family. E3 ligases also bind the substrates, to which they attach activated ubiquitin and polyubiquitin chains. There are an increasing number of E2s and E3s being identified. Unlike E1, these enzymes are specific for a substrate or a group of substrates (Ciechanover, 1998).

Polyubiquitinated proteins are subsequently recognized and degraded by the 26S proteasome. The 26S proteasome is comprised of a core catalytic component, the 20S proteasome, flanked on both sides by a 19S cap. Proteins signaled for degradation are recognized by the 19S cap, enter the cylindrical pore formed by the 20S proteasome, and are degraded by 3 sets of enzymes, with chymotrypsin-like, trypsin-like, and peptidylglutamyl-like activity. Proteasomal-mediated degradation is ATP-dependent. The polyubiquitin chains are hydrolyzed to monomeric ubiquitin through the action of deubiquitinating enzymes. The released monomeric ubiquitin is then recycled in order to attach to other protein substrates (Ciechanover, 1998; Pickart, 2001). In some cases degradation of ubiquitinated proteins may also occur in lysosomes.

**Involvement of the ubiquitin-dependent proteolytic system in PD: Insights from genetics**

PD is usually sporadic. Recently however, cases with specific genetic defects have been identified. First, two distinct mutations were found in the gene encoding for the abundant neuronal presynaptic protein α-synuclein, leading to autosomal dominant PD (Polymeropoulos et al., 1997; Kruger et al., 1998; Clayton and George, 1999). α-synuclein is present within LBs and other intracellular inclusions in a variety of neurodegenerative conditions (Duda et al., 2000; Mezey et al., 2000). Both the wild-type (WT) and the mutant (MT) forms of α-synuclein form fibrillar assemblies in vitro, and the MT forms appear to be more prone to aggregation (Conway et al., 1998; Trojanowski et al., 1998). Transgenic mice or flies expressing WT or MT α-synuclein form intraneuronal synuclein inclusions and demonstrate deficits in the dopaminergic system (Feany and Bender, 2000; Masliah et al., 2000). Co-expression of NAC, a central hydrophobic domain of α-synuclein, with synphilin, a protein that physically interacts with α-synuclein, leads to inclusion formation (Engelender et al., 1999). Such studies have supported the idea that aggregation of α-synuclein is a critical event in PD pathogenesis (Trojanowski et al., 1998; Goldberg and Lansbury, 2000). However, there is no direct evidence that intracellular synuclein aggregation is toxic. WT α-synuclein may even act as a molecular chaperone, preventing the aggregation of other proteins (Souza et al., 2000). The exact role of α-synuclein in aggregate and inclusion formation is therefore uncertain at this point. In particular, it is unknown whether α-synuclein is required for LB-like inclusion formation.

Various studies suggest a link between α-synuclein and the ubiquitin-dependent proteolytic system. α-synuclein levels may be regulated by the proteasome (Bennett et al., 1999; Imai et al., 2000), but this is controversial (Ancolio et al., 2000; Rideout et al., 2001). A glycosylated form of α-synuclein may interact with Parkin, another molecule linked to PD (see below), and may thus be targeted for degradation by the proteasome (Shimura et al., 2001). In addition, α-synuclein is reported to physically interact with tat binding protein 1, a component of the proteasome (Ghee et al., 2000).

A mutation in the gene encoding for Ubiquitin carboxy-terminal hydrolase (UCH-L1) has been identified in one family with autosomal dominant PD (Leroy et al., 1998). UCH-L1 has itself been identified within LBs (Louw et al., 1990). UCH-L1 is an abundant neuronal-specific protein, which belongs to the family of UCHs (Ciechanover, 1998), a large family of deubiquitinating enzymes. The mutation present in this one family led to a 50% loss of UCH-L1 enzymatic activity (Leroy et al., 1998). The cellular substrate(s) of UCH-L1 is/are unknown, but it is thought that it may act on newly synthesized linear proubiquitin or on polyubiquitinated proteins following their degradation by the proteasome (Pickart and Rose, 1985; Ciechanover, 1998; Larsen et al., 1998; Chain et al., 1999). Defects in UCH-L1 may thus lead to accumulation of polyubiquitin chains or of proubiquitin. In a number of contexts polyubiquitin chains inhibit proteasomal activity, presumably by locking onto the proteasome and preventing access of ubiquitinated substrates (Amerik et al., 1997; Piotrowski et al., 1997; Lam et al., 2000; Pickart, 2000). Linear proubiquitin may also inhibit the proteasome (Thrower et al., 2000). Consistent with a role of this enzyme in neurodegeneration, Gad mice, which have a partial deletion of UCH-L1, manifest sensory/motor axia and axonal degeneration, with the formation of ubiquitinated axonal spheroids (Saigoh et al., 1999).

The most common genetic defect identified in PD is a loss of function of the protein Parkin. Inheritance is autosomal recessive, and the majority of defects are deletions (Kitada et al., 1998; Lucking et al., 2000). Affected individuals generally lack Parkin protein expression (Shimura et al., 1999). Interestingly, on pathology no LBs are identified, however WT Parkin has been found in LBs in sporadic PD (Shimura et al., 2001). Parkin functions as an E3 ligase. Deletions or point mutations found in affected families abolish or diminish this activity (Shimura et al., 2000; Zhang et al., 2000). Five substrates for ubiquitination by Parkin, CDCRel-1 (a synaptic protein of the septin family), Parkin itself, synphilin-1, the glycosylated form of α-synuclein mentioned above, and the transmembrane protein Pael receptor have been suggested (Shimura et al., 2000, 2001; Zhang et al., 2000; Chung et al., 2001a; Imai et al., 2001). This genetic defect is therefore the clearest example so far of a dysfunction of the ubiquitin-dependent proteolytic system in PD. The fact that LBs
are not formed in this condition suggests that Parkin may be involved in inclusion formation. This is supported by the fact that it is found within LBs in sporadic PD (Shimura et al., 1999, 2001). The inference is that ubiquitination of certain key substrates of Parkin is required for LB formation.

**Involvement of the ubiquitin-dependent proteolytic system: Insights from sporadic PD**

The genetic information available so far strongly implicates dysfunction of the ubiquitin-dependent degradation system in PD. Cases of sporadic PD, as already mentioned, invariably show ubiquitinated LBs. At least in DLBD, studies with purified CLBs show that the ubiquitin present within the inclusions is in the form of polyubiquitin chains (Iwatsubo et al., 1996). If sporadic LB diseases, like familial PD, are caused by dysfunction of the ubiquitin-dependent degradation system, the defect in the pathway would therefore have to be downstream of the accumulation of polyubiquitinated proteins, i.e., at the level of the proteasome. Of note, immunoreactivity for the proteasome has been identified within LBs (Li et al., 1997). McNaught and Jenner (2001) have found direct evidence for proteasomal dysfunction in PD. In their study, all three proteolytic activities of the proteasome in the substantia nigra were diminished compared to controls. An interesting report by the same group provides a potential basis for this diminution of proteasomal activity: a selective loss of the a subunit of the 20S proteasome was found specifically within melanized neurons of the SNpc in post-mortem PD brain tissue (McNaught et al., 2002a).

**Potential involvement of the ubiquitin-dependent proteolytic system in AD**

Recent studies show decreased proteasomal activity in AD brains (Checler et al., 2000; Keller et al., 2000a). A8, thought to be the major culprit in AD pathogenesis, is known to associate with, and inhibit, the proteasome (Gregori et al., 1995, 1997). In addition, recent reports suggest the existence of a mutant form of ubiquitin, which is not cleaved by C-terminal hydrolases, in AD brains (Ub+) (van Leeuwen et al., 1998). This could be the building block for the formation of processing-resistant polyubiquitin chains, which could inhibit the degradation of other polyubiquitinated substrates, acting in effect as inhibitors of the proteasome (Lam et al., 2000; Lindsten et al., 2002). Presenilins are degraded by the proteasome and their increase in the presence of proteasomal inhibition could account for increased A8 production, leading to further proteasomal inhibition and neuronal degeneration in a feedback loop (Fraser et al., 1998; Layfield 2001). There is therefore substantial evidence that dysfunction of the ubiquitin-dependent proteolytic system may also be involved in AD pathogenesis.

**Relationship between mutant α-synuclein expression and dysfunction of the ubiquitin-dependent proteolytic system**

Given the potential relationship between dysfunction of the ubiquitin-dependent proteolytic system and LB diseases, we decided to investigate whether expression of wild type (WT) or mutant (MT) α-synuclein may be associated with such dysfunction. To this end, we generated stable PC12 cell lines expressing either empty vector, WT, or MT α-synuclein. We found that expression in PC12 cells of A53T MT α-synuclein leads to enhanced granular-vacuolar cellular degeneration both in the naïve and in the neuronally differentiated state (Stefanis et al., 2001a). In contrast, cells expressing WT α-synuclein did not show morphological abnormalities, and were equally sensitive to the apoptotic stimulus of trophic deprivation compared to empty vector control cells (Stefanis et al., 2001a,b). Interestingly, the death induced by MT α-synuclein expression was non-apoptotic, as assessed by nuclear staining. By Electron Microscopy (EM), cell death resembled autophagy (Clarke, 1990; Larsen and Sulzer, 2002). In addition, cells expressing MT α-synuclein showed formation of ubiquitinated cytoplasmic aggregates, that represented polyubiquitinated proteins. In contrast, we did not find any evidence for α-synuclein aggregation in the MT α-synuclein-expressing lines. These abnormalities were accompanied by a decrease of proteasomal and lysosomal function (Stefanis et al., 2001a). Another significant finding was that cells expressing MT α-synuclein showed complete absence of evoked dopamine release, likely due to the loss of the dopamine-storing and -secreting dense core granules, as visualized by EM. These findings have led us to propose the following model for the aberrant effects of mutant α-synuclein expression: this stimulus leads to dysfunction of the ubiquitin-dependent proteolytic system, perhaps in parallel with a dysfunction of the lysosomal system. Such dysfunction leads to accumulation of ubiquitinated aggregates and autophagy and, either directly or indirectly, to cellular dysfunction and cell death (Stefanis et al., 2001a). Another group has also found an association between proteasomal dysfunction and inducible expression of the other MT α-synuclein, A30P, in PC12 cells (Tanaka et al., 2001). In the same study, MT α-synuclein-expressing PC12 cells were found to be more vulnerable to proteasomal inhibition compared to WT-expressing cells (Tanaka et al., 2001). This was also the case in another study with a different neuronal cell type (Lee et al., 2001) In conjunction, our own studies and those of others suggest a link between MT α-synuclein expression and dysfunction of the ubiquitin-dependent proteolytic system at the level of the proteasome. Our findings also raise the possibility that the effects of α-synuclein on aggregate formation may be indirect, through proteasomal dysfunction, and may not require its presence within the aggregates. This is supported by another study, in which α-synuclein
promoted aggregation of mutant huntingtin, but did not colocalize with the aggregates (Furlong et al., 2000).

**Post-mitotic cortical neurons treated with proteasomal inhibitors: A model system to study the neuronal effects of dysfunction of the ubiquitin-dependent proteolytic system**

Given the potential involvement of the ubiquitin-dependent proteolytic system, and in particular of the proteasome, in LB disease pathogenesis, as mentioned above, and highlighted in recent opinion-reviews (Chung et al., 2001b; McNaught et al., 2001; Kruger et al., 2002) we have embarked on a series of studies to test whether such dysfunction could account for the neuropathological features of the disease. The availability of specific pharmacological inhibitors of the proteasome has allowed us to examine this question in model neuronal cell culture systems. Pharmacological inhibition of the proteasome induces apoptotic death in a variety of cell types, including primary neurons (Pasquini et al., 2000; Qiu et al., 2000). Proteasomal inhibitors also potentiate the formation of cytoplasmic and nuclear inclusions induced by over-expression of mutant polyglutamine repeat or other misfolded proteins (Cummings et al., 1998; Klement et al., 1998).

We first examined the effects of proteasomal inhibition on cultured embryonic cortical neurons, which provide a rich source of primary post-mitotic neurons that are affected in LB diseases (Spillantini et al., 1998; Mattila et al., 2000). In agreement with previous work (Cummings et al., 1998), we found that the specific proteasomal inhibitors lactacystin (Lact) (Fenteany and Schreiber, 1998), ZIE[Ot-Bu] -A-leucinal (PSI) (Figueiredo-Pereira et al., 1994) and epoxomicin (Meng et al., 1999), induced apoptotic death of cultured E18 primary cortical neurons. Proteasomal inhibition-induced death was prevented by Actinomycin D and BAF, indicating that transcription and caspase activation are both required for this type of neuronal apoptotic death. We also found that a proportion of neurons contained discrete cytoplasmic regions of ubiquitin immunoreactivity, consistent with cytoplasmic inclusions. Importantly, inclusions were seen only in viable neurons (Rideout and Stefanis, 2002). To examine further the relationship between inclusion formation and death, we assessed whether agents that were protective affected ubiquitinated inclusion formation. We found that Actinomycin D, but not BAF, prevented inclusion formation. The results with BAF provide a dissociation between inclusion formation and death, and suggest that these two cellular processes are not inextricably linked. The effect of Actinomycin D suggests that induction of certain genes is needed to organize aggregated proteins into discrete inclusions (Rideout and Stefanis, 2002).

α-synuclein is an integral component of LBs (Spillantini et al., 1997, 1998). Accordingly, we also found co-localization of α-synuclein within cytoplasmic ubiquitinated inclusions formed following exposure to proteasomal inhibitors in primary cortical neurons. This co-localization suggested to us that aggregated, possibly ubiquitinated, α-synuclein may accumulate within these inclusions. However, by Western immunoblot and by co-immunoprecipitation, we did not detect ubiquitinated or oligomerized α-synuclein or a change in total protein levels (Rideout and Stefanis, 2002). Based on the lack of evidence for α-synuclein aggregation or ubiquitination, it is conceivable that endogenous rat α-synuclein in our model acts as a molecular chaperone, as has been shown in vitro for its human homologue (Souza et al., 2000). We also immunostained proteasomal inhibitor-treated cortical neuron cultures with a variety of other antibodies to investigate whether components of LBs are also present in these inclusions, and whether such accumulation is specific. The panel of proteins screened thus far appears in Table 1. Of particular interest is the presence, as in LBs (Goldman et al., 1983; Auluck et al., 2002) of the molecular chaperone Hsp-70 and the neuronal intermediate filament α-interneulin in the majority of ubiquitinated inclusions. The co-localization of HSP-70 in particular within the inclusions supports the idea that the accumulation of α-synuclein in these inclusions may reflect its role as a molecular chaperone. Also of note is the positive labeling of ubiquitin- or α-synuclein-positive inclusions with Thioflavin-S, as in LBs (Hashimoto et al., 1998), indicative of a β-pleated sheath, fibrillar structure. Therefore, at the immunocytochemical level, the inclusions seen in cortical neurons following proteasomal inhibition resemble cortical LBs of PD, DLBD and LBVAD (Rideout and Stefanis, 2002).

The molecular pathways underlying inclusion formation, and the role of ubiquitination in particular, are unclear. To address this issue in our model, we used a dominant negative (DN) form of the E2 Cdc34, which specifically inhibits the Cdc34-dependent ubiquitination pathway (Banerjee et al., 1995). This same DN prevented the formation of intranuclear inclusions induced by

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<th>PROTEINS/HISTOCHEM.</th>
<th>POSITIVE/NEGATIVE</th>
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<tr>
<td>Ubiquitin</td>
<td>+</td>
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<td>α-synuclein</td>
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<td>Hsp-70</td>
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<td>α-interneulin</td>
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<td>γ-tubulin</td>
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<td>β-tubulin</td>
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<td>Thioflavin S</td>
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<td>Parkin</td>
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<td>Bax</td>
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<td>GAPDH</td>
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<td>β-actin</td>
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<td>Cyclin D1</td>
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<td>Cyclin E</td>
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<td>p53</td>
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<td>ERK-2</td>
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<td>20S Proteasome</td>
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expression of truncated MT Huntingtin (Saudou et al., 1998). Overexpression of Cdc34-DN, but not WT in cortical neurons, almost completely prevented formation of cytoplasmic ubiquitinated inclusions induced by inhibition of the proteasome, suggesting that Cdc34-dependent ubiquitination of certain protein substrates is required for inclusion formation in this model (Rideout and Stefanis, 2002). These results are consistent with the idea that ubiquitination of certain proteins is involved in inclusion formation, and with studies showing absence of LBs in PD patients with parkin mutations (Shimura et al., 1999), in whom ubiquitination is defective. We then asked whether Cdc34-DN could also modulate Lact-induced neuronal cell death. We found that Cdc34-DN, but not Cdc34-WT-expressing neurons, were significantly protected from Lact-induced apoptosis (Rideout and Stefanis, 2002).

The fact that cdc34-DN inhibited inclusion formation and was also partially protective against Lact-induced apoptotic death suggests either that the inclusions themselves are in fact toxic to cortical neurons, or that it is the process of ubiquitination of specific substrate proteins that is involved in the toxicity. Regarding this second possibility, it has recently become apparent that ubiquitination of protein substrates may not only serve to target them for degradation, but may also inactivate them or confer properties that influence their signaling potential (Hicke, 2001). Cdc34 is known to participate in the ubiquitination, amongst other proteins, of cell cycle molecules (Banerjee et al., 1995), which may in turn affect cell death pathways (Park et al., 1997). It is possible that cdc34-DN, by preventing ubiquitination of such substrates, may alter cell cycle signaling elicited by proteasomal inhibition. Our own data suggest cell cycle involvement in this apoptotic pathway (Rideout et al., 2003). For a schematic overview of these findings see Figure 1.

Inclusion formation and neuronal death in ventral midbrain cultures following disruption of the ubiquitin-dependent proteolytic system

Although LBs are being increasingly identified in cortical regions, even in brains of patients with idiopathic PD, they are invariably found in dopaminergic neurons of the ventral midbrain in PD patients (Fahn, 1995; Gibb, 1993; Spillantini et al., 1997). It is therefore important to investigate whether such neurons may also form inclusions and undergo cell death in response to perturbations of the ubiquitin-dependent proteolytic system. In a recent study by McNaught et al., application of 5-10 µM Lact to rat ventral midbrain cultures led to marked loss of TH staining and the formation of cytoplasmic ubiquitin/a-synuclein-positive inclusions. Interestingly, the GABAergic population was relatively spared (McNaught et al., 2002b). In this study, however, the phenotype of cells showing inclusion formation was not investigated, and the occurrence of cell death, as opposed to a mere loss of dopaminergic phenotype, was not shown. Our own preliminary findings suggest that pharmacological proteasomal inhibition induces apoptotic death of ventral midbrain dopaminergic neurons, and that ubiquitinated inclusions can be identified within phenotypically defined dopaminergic neurons (Rideout and Stefanis, unpublished results).

Interestingly, in the same study, a pharmacological inhibitor of deubiquitination also induced loss of TH immunoreactivity and formation of α-synuclein-positive inclusions, thus linking defects of deubiquitination with inclusion formation. This is important, in view of the genetic data linking defects of UCH-L1 with PD and neurodegeneration (Leroy et al., 1998; Saigoh et al., 1999).

The authors’ findings led them to suggest that nigral dopaminergic neurons may be more sensitive to proteasomal dysfunction compared to other cells, and that this may provide the basis for their selective vulnerability in PD (McNaught et al., 2002b). We believe that, if proteasomal dysfunction plays a role in PD pathogenesis, it is more likely that selective proteasomal dysfunction in nigral neurons may account for their selective vulnerability. This is based on the fact that relatively similar concentrations of proteasomal inhibitors induce death in neurons and other cell types (Drexler, 1997; Pasquini et al., 2000; Qiu et al., 2000; Rideout and Stefanis, 2002), and on the previously mentioned finding of loss of proteasomal subunits selectively within melanized nigral neurons (McNaught et al., 2002a). Proteasomal dysfunction in nigral neurons could occur because of genetic or environmental factors that have been linked to PD, such as oxidative stress, aging, or protein aggregation (Keller et al., 2000b,c; Bence et al., 2001; Jana et al., 2001).

Conclusions

It has generally been thought that inclusions in neurodegenerative conditions, and, in particular, in LB
diseases, are formed because of abnormal protein folding. The possibility that abnormal protein degradation may participate in the formation of such inclusions has received less attention. There is now increasing evidence for involvement of the ubiquitin-dependent proteolytic system, and in particular of the multicatalytic proteasome complex, in LB diseases. Recent studies suggest that proteasomal dysfunction can lead to apoptotic cell death and inclusion formation in neurons vulnerable in these disorders. Proteasomal dysfunction may be a primary initiating factor in the disease process, for example following expression of MT α-synuclein. Alternatively, proteasomal dysfunction may be a contributing, potentiating factor in inclusion formation and neuronal death. The relationship between inclusion formation and neuronal dysfunction and death remains controversial (Sisodia, 1998). Results from the model of proteasomal inhibition of cultured cortical neurons suggest that inclusion formation and neuronal death share common mechanisms, such as requirement for ubiquitination of specific substrates and transcription, but can also be dissociated at a certain level.

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