Proteasomal dysfunction may underlie certain neurodegenerative conditions such as Parkinson disease. We have shown that pharmacological inhibition of the proteasome in cultured neuronal cells leads to apoptotic death and formation of cytoplasmic ubiquitinated inclusions. These inclusions stain for α-synuclein and assume a fibrillar structure, as assessed by thioflavine S staining, and therefore resemble Lewy bodies. α-Synuclein is thought to be a central component of Lewy bodies. Whether α-synuclein is required for inclusion formation or apoptotic death has not been formally assessed. The present study examines whether α-synuclein deficiency in neurons alters their sensitivity to proteasomal inhibition-induced apoptosis or inclusion formation. Cortical neurons derived from α-synuclein-null mice showed a similar sensitivity to death induced by the proteasomal inhibitor lactacystin compared with neurons derived from wild-type mice. Furthermore, the absence of α-synuclein did not influence the percentage of lactacystin-treated neurons harboring cytoplasmic ubiquitinated inclusions or alter the solubility of such inclusions. In contrast, however, ubiquitinated inclusions in α-synuclein-deficient neurons lacked amyloid-like fibrillization, as determined by thioflavine S staining. This indicates that although α-synuclein deficiency does not affect the formation of ubiquitinated inclusions, it does significantly alter their structure. The lack of effect on survival in α-synuclein knock-out cultures further suggests that the fibrillar nature of the inclusions does not contribute to neuronal degeneration in this model.

Since the identification of two α-synuclein mutations that are linked to the development of autosomal dominant Parkinson disease (PD)1 (1, 2), considerable effort has been made to characterize both the normal function of this protein and its role in the pathogenesis of PD and related synucleinopathies. Additional genetic abnormalities have since been described in patients with PD, including mutations in the genes encoding for Parkin (3) and UCH-L1 (4). These two proteins are notable in that they are both involved in proteasomal-dependent degradation of ubiquitinated proteins, linking dysfunction of ubiquitin-dependent protein clearance to the pathogenesis of PD (5). Parkin has E3 ligase activity, which is sensitive to PD-linked mutations (6). UCH-L1 is a neuronal-specific protein that mediates the de-ubiquitination of proteins and the cleavage of pro-ubiquitin polypeptides into monomeric ubiquitin (4, 7). More recent data suggest that the main function of UCH-L1 is to stabilize monomeric ubiquitin (8). Another report suggests that UCH-L1 has an additional E3 ligase activity that may be more relevant to its relationship to PD (9). In addition, α-synuclein may associate with the 19 S or the 20 S proteasome (10–12), an association that may be responsible for the reported effects of α-synuclein on proteasome function in cellular systems (13–15).

α-Synuclein is an abundant protein of unclear function. It is a natively unfolded protein and aggregates in vitro following an ordered transition to an oligomeric protofibril and ultimately to a fibrillar β-pleated sheet structure. Regions within α-synuclein have been identified that mediate or are necessary for such oligomerization or aggregation of the protein (16–18). α-Synuclein is a major component of Lewy bodies (LBs) in PD, and purified LBs have been shown to contain aggregated α-synuclein (19, 20). Despite its abundance within LBs, it is, however, not clear whether α-synuclein is required for their formation or is involved in mediating toxicity associated with these structures.

The data concerning the role of α-synuclein in cell death are somewhat contradictory. Several groups have reported that overexpression of mutant and, in some cases, wild-type (WT) α-synuclein directly induces death of various cell types (e.g. see Refs. 21–24). In contrast, several reports suggest a survival-promoting activity of WT or even mutant α-synuclein (25–27). Therefore, the potential role of α-synuclein in cell death pathways remains unclear.

In addition to genetic data, pathological evidence indicates that proteasomal dysfunction occurs in the substantia nigra of PD patients (28, 29). We have used pharmacological inhibitors of the proteasome to model such pathological features of PD. Proteasomal inhibition of cultured rat cortical neurons induces apoptotic death (30, 31) and the formation of cytoplasmic inclusions that contain, among other proteins, ubiquitin and α-synuclein (31). Cell death and inclusion formation have also been observed in dopaminergic PC12 cells (32) as well as cultured ventral midbrain neurons (33) and in vivo following infusion of the proteasomal inhibitor lactacystin into the substantia nigra (34). An important feature of the cellular inclusions induced by inhibition of the proteasome is that they possess a fibrillar structure reminiscent of LBs and other inclusions, as revealed by the fluorescent histochemical marker thioflavine S (31). The formation of ubiquitinated inclusions in
α-Synuclein Mediates Fibrillization of Inclusions

this model is a regulated process requiring ubiquitination of specific substrates and transcription (31). However, as with LbSs, it is not clear whether α-synuclein is required for their formation.

In the present work, we address the following questions. 1) What is the conformation of α-synuclein within cortical neuronal inclusions following proteasome inhibition? 2) What is the effect of lack of α-synuclein on the formation, solubility, and structure of such inclusions? 3) Does lack of α-synuclein alter the sensitivity of cortical neurons to proteasomal inhibition-induced death?

EXPERIMENTAL PROCEDURES

Cell Culture and Genotyping—Rat embryonic cortical neurons were prepared as described (31, 35). α-Synuclein +/-/+/- or +/+ neurons (36) were obtained from embryonic day 16 mouse embryos resulting from heterozygous matings as described (37). Genotyping was performed on each individual embryo by PCR from tail DNA. The primers used were as follows: 1) SYN6591, TCA CAC TTA CAC GAC GTG G and 2) SYN7005, GTC CCT GTT GTC TGC GAG C to detect the wild-type allele, and 3) synNEO, ATG GAA GGA TTG GAG CTA CGG to detect the targeted allele.

Induction of Neuronal Death—Cortical neurons were cultured for 3 or 10 days in vitro (DIV) before application of reagents. The reason these two time points were selected was that, as we have previously reported, there is a marked induction of the levels of α-synuclein during this period in rat cortical neuron cultures (38). A similar induction is seen between DIV 3 and DIV 10 in embryonic day 16 mouse cortical neurons (data not shown). On DIV 3 or 10, the inhibitor of the 20 S proteasome lactacystin (39) was added to the cultures. At indicated times following addition of lactacystin (10 μM) to the cultures, cells were lysed and neuronal viability was estimated using counts of intact nuclei as described previously (35). Total surviving neurons are expressed as the percentage of untreated cultures at the time of cell lysis. In parallel experiments, the percentage of apoptotic nuclei, as an index of cell death, was assessed using the nuclear dye Hoechst 33342 (Sigma, 1 μg/ml) as described previously (35).

Immunofluorescence—Neurons grown on glass coverslips were fixed in freshly prepared 3.7% formaldehyde for 25 min at 4 °C and then incubated with 10% normal goat serum with 0.4% Triton X-100 to block nonspecific binding, followed by incubation with the primary antibody for 1 h at room temperature. Specific antibodies used were rabbit anti-ubiquitin (1:100; Dako), mouse anti-α-synuclein (1:200; Affinity Bioreagents), or mouse anti-synuclein-1 (1:50; Transduction Laboratories). Following incubation with fluorescent secondary antibodies (Cy2, 1:100 or Cy3, 1:250; Jackson ImmunoResearch), coverslips were placed on glass slides and visualized using standard epifluorescence or confocal microscopy (Ziess LSM410). For thioflavine S staining, freshly fixed cells were incubated with 0.05% thioflavine S in phosphate-buffered saline (Sigma), washed for 5 min three times with 80% ETOH, and then blocked for subsequent immunostaining.

For counts of ubiquitin- and thioflavine S-positive cytoplasmic inclusions, stained coverslips were observed by a rater blinded to both the genotype and the experimental condition using standard epifluorescence or confocal microscopy. From three to four coverslips per individual embryo, 100 cells were observed each and assessed for the presence of discrete ubiquitin- or thioflavine S-positive cytoplasmic inclusions as described previously (31). The data were pooled from embryos of the same genotype for analysis.

For in situ extraction of soluble protein (31), cells were incubated with detergent-containing extraction buffer (85 mM PIPEs, pH 6.94, 10 mM EGTA, 1 mM MgCl2, and 0.1% Triton X-100, supplemented with protease inhibitor mixture (Roche) for 10 min at room temperature, washed in phosphate-buffered saline, and fixed as above with 3.7% formaldehyde for 25 min at 4 °C. The fixed cells were then processed as described for anti-ubiquitin. We have previously shown that proteasome inhibitor-treated rat cortical neurons show insoluble ubiquitinated inclusions that are resistant to such extraction (31).

Western Immunoblotting—Rat cortical neurons exposed at DIV 3 or DIV 10 to lactacystin (10 μM) for 30 h were incubated, as described above, with detergent-containing extraction buffer at room temperature for 10 min. The soluble extracted material in the buffer was removed, and the remaining insoluble material was scraped in the same buffer and solubilized by sonication. The samples were diluted in SDS-sample buffer containing 5% β-mercaptoethanol and subjected to polyacrylamide gel electrophoresis. The separated proteins were transferred to nitrocellulose membranes and incubated with a mouse monoclonal anti-synuclein 1 antibody (BD Transduction Laboratories; 1:1000) or rabbit anti-α-synuclein (1:500; generously provided by Dr. Janetta Culvenor, University of Melbourne) (40) overnight at 4 °C. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary anti-mouse or rabbit antibodies (Pierce; 1:10000) and developed with SuperSignal West (Pierce). Equal protein loading was assessed by Ponceau S staining or stripping of the membranes and probing for mouse anti-β-actin (Sigma; 1:20,000).

RESULTS

α-Synuclein Accumulates within Insoluble Inclusions in Pro teaseosome Inhibitor-treated Rat Cortical Neurons—We have previously demonstrated that the ubiquitinated inclusions formed following proteasomal inhibition resist in situ extraction by a buffer containing 0.1% Triton X-100 (31). To investigate whether the α-synuclein that we had identified within such inclusions would also be detergent-insoluble we performed similar in situ extraction of embryonic day 18 rat cortical neurons cultured for 10 days (DIV 10) and then treated with lactacystin (10 μM, 30 h) or vehicle (control). Cultures were then fixed and immunostained for anti-ubiquitin and anti-α-synuclein. Control cultures of rat embryonic cortical neurons showed little cytoplasmic ubiquitin and α-synuclein immunostaining following in situ extraction (Fig. 1A, top panel). In contrast, lactacystin-treated cultures showed a dramatic accumulation of ubiquitin immunoreactivity distributed diffusely throughout the cytoplasm (not shown) or organized within discrete cytoplasmic inclusions (Fig. 1A, bottom panel, arrow) as we have reported previously (31). Many of the ubiquitin-positive inclusions also showed clear α-synuclein co-localization (Fig. 1A, arrow). Similar results were obtained with DIV 3 cultures (data not shown).

Oligomerization of Detergent-insoluble α-Synuclein following Inhibition of the Proteasome—The results presented above indicate that α-synuclein accumulates within detergent-resistant inclusions in proteasome inhibitor-treated neurons. To determine the nature of α-synuclein within such inclusions, we performed Western immunoblot analysis of the detergent-insoluble fractions. Using a mouse monoclonal antibody raised against synuclein-1, we observed a reduction in the 15-kDa α-synuclein monomer band (Fig. 1B, asterisk) in lactacystin-treated samples. Upon longer exposure of the film we observed two higher molecular weight (HMW) bands in lysates derived from lactacystin-treated neurons migrating at Mr ~45,000–50,000 and 65,000–70,000. These bands are present in multiple independent samples derived from lactacystin-treated cultures (Fig. 1B, arrows). The sizes of the HMW α-synuclein immunoreactive bands do not indicate ubiquitination, which would be represented by multiple bands separated by Mr ~8,000. It is likely that the bands we have identified with this antibody represent oligomeric forms of α-synuclein, in particular a trimer and a tetramer. Similar findings were obtained with DIV 3 cortical neuronal cultures (data not shown).

To confirm the formation of the HMW oligomeric α-synuclein species, we utilized a second antibody raised against the C terminus of human α-synuclein. The monomeric form of α-synuclein is indicated by an asterisk in Fig. 1C, whereas the HMW oligomeric species are indicated by brackets. This α-synuclein antibody provided an increased sensitivity in detecting the HMW oligomeric species compared with the mouse monoclonal antibody (compare Fig. 1B and 1C). There are three apparent clusters of HMW α-synuclein oligomers in lactacystin-treated detergent-insoluble fractions migrating at Mr ~31,000–35,000, 43,000–49,000, and 65,000–70,000 (Fig. 1C). These clusters confirm the oligomerization of α-synuclein revealed by the mouse monoclonal synuclein-1 antibody. The additional bands detected by this antibody may represent other oligomeric forms of α-synuclein.
post-translational modifications such as phosphorylation. We conclude that α-synuclein is at least partially present in an oligomeric form within detergent-insoluble inclusions in neurons treated with lactacystin.

Deletion of α-Synuclein Does Not Alter Neuronal Sensitivity to Apoptotic Stimuli—Given the relationship between α-synuclein and the proteasome as well as the reported effects of α-synuclein on the apoptotic pathway, we wished to determine whether lack of α-synuclein would have an effect on neuronal survival/death following exposure to lactacystin. To this end, we used cultures derived from WT or knock-out (KO) α-synuclein mice (36). There was no difference in survival, as assessed by counts of intact nuclei (Fig. 2A) or apoptosis, as assessed by counts of apoptotic nuclei (Fig. 2B) between WT and α-synuclein KO cultured cortical neurons.

To investigate whether the lack of effect of deleting α-synuclein was specific for the apoptotic stimulus of proteasomal inhibition, we also tested the effects of the DNA-damaging agent camptothecin on these cultures. We and others have previously reported that this agent induces apoptotic death of postmitotic neurons (35, 41). Again, there was no difference in survival or apoptosis between WT and α-synuclein KO neurons (Fig. 2, A and B). We conclude that deleting α-synuclein has no significant impact on survival of embryonic cortical neurons exposed to apoptotic stimuli and, in particular, to proteasomal inhibition.

Absence of α-Synuclein Does Not Alter the Number or the Solubility of Ubiquitinated Inclusions Formed following Lactacystin Treatment—Given the correlation between α-synuclein and cytoplasmic inclusions, we wished to investigate whether its presence was required for inclusion formation in this model. We therefore treated α-synuclein WT and KO cortical neurons with lactacystin and 16 and 24 h later assessed the percentage of neurons that showed cytoplasmic ubiquitinated inclusions. There was no difference between the two genotypes (Fig. 3).

The possibility existed that α-synuclein may not alter the initial formation of the inclusions but may alter their subsequent solubility through its aggregating properties. Using the same in situ detergent extraction method as in Fig. 1, we were unable to find a significant difference between the two genotypes in terms of detergent-insoluble ubiquitinated inclusions (Fig. 4, A and B).

We conclude that α-synuclein does not influence the formation or solubility of ubiquitinated inclusions formed following proteasomal inhibition.

Absence of α-Synuclein Alters the Fibrillar Nature of Ubiquitinated Inclusions—We have previously shown that in cultured rat cortical neurons exposed to the proteasome inhibitor lactacystin, both ubiquitin- and α-synuclein-positive inclusions are labeled with thioflavine S, indicating that the inclusions are fibrillar in composition (31). This feature is also present in pathological inclusions such as LBs (42). The fibrillar nature of these inclusions is important, given that it is presumably because of the presence of misfolded protein(s), which are increasingly linked to neurodegeneration of various etiologies (e.g. see Ref. 43). To determine whether this is also the case in cultured mouse cortical neurons and to what degree these markers co-localize, we performed double immunostaining for ubiquitin and/or α-synuclein with thioflavine S in wild-type neurons treated with lactacystin. We found that ~70% of ubiquitin-positive inclusions also contained thioflavine S staining and that ~60% of ubiquitin-positive inclusions also contained α-synuclein immunoreactivity.

To determine whether the absence of α-synuclein would alter this fibrillar structure of the inclusions, we performed ubiquitin immunostaining and thioflavine S staining in WT and

**Fig. 1.** α-Synuclein accumulates within detergent-insoluble ubiquitinated inclusions and higher molecular weight species following inhibition of the proteasome. Primary rat embryonic cortical neurons at 10 DIV were exposed to vehicle (control (Ctrl.)) or lactacystin (10 μM, 30 h), incubated with extraction buffer containing 0.1% Triton X-100 for 10 min, and either fixed (A) or separated by SDS-PAGE (B). In A, fixed cells were double immunostained for ubiquitin (red) and synuclein-1 (green). Note the co-localization of α-synuclein and ubiquitin within a cytoplasmic inclusion (arrow) remaining following detergent extraction. In parallel wells, the remaining detergent-insoluble material was collected and separated by SDS-PAGE, and the membranes were probed with mouse anti-synuclein-1 (B) or a rabbit anti-α-synuclein antibody (C). The upper panel in B is a short exposure showing a decrease in the monomeric form of α-synuclein (asterisk). The lower panel in B is the same membrane exposed for a longer period to reveal several higher molecular weight α-synuclein-positive species (arrows) in lactacystin-treated samples. In C, monomeric α-synuclein is indicated by an asterisk, and the HMW species are indicated by braces. Three independently treated samples are shown for each antibody.
**DISCUSSION**

A major focus of the research relating to synuclein has centered on its ability to oligomerize, aggregate, and form inclusions. However, whether these events are primary and whether they are responsible for the formation of the ubiquitinated inclusions observed in PD and other neurodegenerative diseases is not known. We have found detergent-resistant oligomeric species of endogenous α-synuclein following proteasomal inhibition of cultured cortical neurons (Fig. 1B). The detergent buffer we have used solubilizes cyttoplasmic α-synuclein but not α-synuclein present within the ubiquitinated inclusions (Fig. 1A). Therefore, the identified oligomeric forms of α-synuclein, which only appear following proteasomal inhibition, are derived from the cytoplasmic inclusions. Such oligomeric species were not appreciated when we examined total or fractionated lysates that had been solubilized in stronger detergents (31). It is likely that the in situ extraction method that we have used here has enabled us to identify these oligomeric forms. Taken together, these results suggest that treatment of cortical neurons with lactacystin does not lead directly to the formation of oligomeric species of α-synuclein, but rather that such oligomeric species are formed at a subsequent stage within the inclusions. Alternatively, these data may suggest that oligomeric forms of synuclein are stabilized by interactions with other proteins within the inclusions.

What could be the mechanism responsible for the formation of such oligomeric species? A likely possibility is molecular crowding, the increased concentration of molecules, in particular proteins, within a restricted space of the cell. Molecular crowding is known to lead to an increased propensity to aggregation (43) and should occur within cytoplasmic inclusions in which a number of intracellular proteins accumulate (5, 31). α-Synuclein itself has been shown to aggregate more rapidly under conditions of molecular crowding in in vitro experiments (44).

Further support for the idea that α-synuclein oligomerizes and aggregates within the inclusions comes from our experiments with cortical neuron cultures derived from α-synuclein knock-out mice. Oligomerization of α-synuclein is the first step that leads to the subsequent formation of amyloid-like fibrillar...
structures that stain positive with thioflavine S (18, 42). We have indeed observed such positive staining in inclusions formed in rat and mouse neurons following proteasomal inhibition (Fig. 5) (91). Neurons that lack α-synuclein, however, do not demonstrate such labeling (Fig. 5). Notably, lack of α-synuclein does not alter the formation or solubility of the inclusions (Figs. 3 and 4). It appears, therefore, that α-synuclein is required for the fibrillar nature of the inclusions but not for their formation. In conjunction with our previous results, these data lead to the following model of inclusion biogenesis following proteasomal inhibition. Ubiquitinated inclusions are initially formed through a process that requires first Cdc42 ubiquitin conjugation activity and then novel or ongoing transcription (91). The genes that regulate this process are unknown. Subsequent to the formation of inclusions, α-synuclein oligomerizes and leads to the formation of fibrillar, amyloid-like structures within the inclusions.

At this point it is unclear whether such mechanisms operate to generate inclusions in PD or other neurodegenerative conditions. Our results, however, do show that the observed oligomerization and aggregation of α-synuclein in a variety of such conditions need not be construed as a primary event but rather may be the consequence of the incorporation of α-synuclein within inclusions.

A number of studies have reported a role for α-synuclein in the control of apoptotic pathways, but only Ostrerova et al. (21) have addressed whether it is required for apoptosis to occur. They found that down-regulation of α-synuclein via antisense oligonucleotides led to a decrease of serum deprivation-induced apoptosis of human embryonic kidney 293 cells. In contrast, we have found no difference between WT and α-synuclein-null neurons in the rate of apoptotic death following proteasomal inhibition or DNA damage. Our studies were performed in primary neurons, and we believe that they more closely reflect the physiologic function of α-synuclein, especially because they are consistent with data from the knock-out mice, in which there are no obvious abnormalities in the number of neurons in the central nervous system (36, 45).

Previous reports have shown that overexpression of mutant (13, 46) or even WT α-synuclein (46) in dopaminergic cell lines increases susceptibility to proteasomal inhibition-induced apo-
The lack of an effect on apoptotic death, when viewed together with the effect on the fibrillar nature of the inclusions, raises another interesting point: the neurons die irrespective of the fibrillar structure of the inclusions and the presence of oligomeric species of α-synuclein. There are a number of interpretations for this observation. 1) The oligomeric species of α-synuclein and the resultant fibrillar forms are sequestered within the inclusions and therefore do not have a deleterious effect on the neurons because they are denied free access to the cytoplasm. 2) Oligomeric α-synuclein and fibrilization could potentially be toxic, even when present within inclusions, but the neurons die through other more direct mechanisms such as activation of the cell cycle machinery (47) or of p53 (37). 3) Oligomeric α-synuclein and fibrillar inclusions are not toxic to the neurons.

In conclusion, we have demonstrated that α-synuclein is required for the fibrillar nature but not the initial formation of ubiquitinated inclusions following proteasomal inhibition of cultured cortical neurons. This, to our knowledge, is the first time that α-synuclein has been identified as the factor responsible for the fibrillar nature of intracellular inclusions. This excludes reports in which α-synuclein itself is overexpressed and thus is the obvious causative factor in fibrillar aggregate formation. Fibrillar structures within inclusions are likely caused by the oligomerization of α-synuclein, which we have found to occur within the inclusions. α-Synuclein did not have an effect on apoptotic death in this model or in another classical model of apoptotic neuronal death, indicating that α-synuclein is not an integral component of apoptotic pathways in neurons and further suggesting a dissociation between inclusions and death in this model.
