

# Proteasome inhibition induces reversible impairments in protein synthesis

Qunxing Ding,\* Edgardo Dimayuga,\* William R. Markesbery,\*<sup>#</sup> and Jeffrey N. Keller\*<sup>·§,1</sup>

\*Sanders-Brown Center on Aging, <sup>#</sup>Department of Neurology and Neuropathology, <sup>§</sup>Department of Anatomy and Neurobiology, University of Kentucky, Lexington, Kentucky, USA

**ABSTRACT** Proteasome inhibition occurs during normal aging and in a variety of age-related diseases, with inhibition of proteasome function sufficient to induce physiological and pathological alterations observed in each of these conditions. It is presumed that proteasome inhibition induces cellular alterations by promoting rapid protein accumulation, as the direct result of impairments in protein removal, which assumes protein synthesis remains relatively unchanged during proteasome inhibition. We conducted experimentation using established proteasome inhibitors and primary rat neuron cultures in order to elucidate whether proteasome inhibition had any effect on neuronal protein synthesis. Proteasome inhibition impaired neuronal protein synthesis, with concentrations of inhibitor necessary to significantly inhibit protein synthesis similar to the concentrations necessary to induce subsequent neuron death. The inhibition of protein synthesis was reversible during the first 6 h of treatment, with the neurotoxicity of proteasome inhibition reversible during the first 12 h of treatment. These studies are the first to demonstrate a potentially important interplay between the proteasome and protein synthesis in neurons, and the first to identify that some effects of proteasome inhibition are reversible in neurons. Together these findings have important implications for understanding proteasome inhibition as a potential contributor to aging and age-related disease.—Ding, Q., Dimayuga, E., Markesbery, W. R., Keller, J. N. Proteasome inhibition induces reversible impairments in protein synthesis. *FASEB J.* 20, 1055–1063 (2006)

*Key Words:* aging • Alzheimer's disease • neurodegeneration • Parkinson's disease • protein aggregation

THE PROTEASOME IS a large intracellular protease that is responsible for degrading most short-lived proteins and a large number of misfolded and aggregate prone proteins (1, 2). The interest in proteasome biology was dramatically elevated when studies began to report that proteasome inhibition occurred during aging in most cells and tissues (3, 4) and in a large number of neurodegenerative disorders, including Alzheimer's disease (4, 5), Parkinson's disease (PD) (4, 6), and

Huntington's disease (4, 7). The development of specific inhibitors of proteasome function (8, 9) has allowed for numerous studies to identify that proteasome inhibition is sufficient to induce numerous neurochemical and neuropathological abnormalities observed in aging and age-related neurodegenerative disorders (4, 10–16). Current dogma suggests that inhibition of proteasome function promotes cellular toxicity by promoting the rapid accumulation of proteins in cells due to an impairment in their removal, which then inevitably leads to the development of neuropathology and neuron death. In this model, protein synthesis is presumed to remain relatively unchanged in response to proteasome inhibition.

Protein synthesis is a continual and essential cellular function that is mediated by the numerous ribosomal complexes present throughout the cytosol, with ribosomes responsible for translating messenger RNA (mRNA) into the different proteins necessary for cell homeostasis. The rate of protein synthesis can be regulated at many different levels ranging from alterations in the levels of ribosome complexes, altering ribosomal performance, and altering the availability of RNA molecules. An increasing number of studies have suggested that there may be a link between the rates of protein synthesis and alterations in proteasome function. For example, studies have demonstrated that proteasomes degrade ribosomal proteins (17) and proteins involved in regulating translation (18, 19). Studies have demonstrated that decreases in ribosome function and protein synthesis occur in aging (20, 21) and Alzheimer's disease (22), two conditions associated with proteasome inhibition. While these findings suggest there may be a potential interplay between protein synthesis and the proteasome, it is unclear whether proteasome inhibition is capable of promoting impairments in neuronal protein synthesis or if there is any relationship between alterations in protein synthesis and the subsequent toxicity of proteasome inhibition.

In the present study we sought to elucidate whether proteasome inhibition had any effect on neuronal

<sup>1</sup>Correspondence: 205 Sanders-Brown, 800 S. Limestone, University of Kentucky, Lexington KY 40536-0230, USA. E-mail: jnkell0@uky.edu  
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protein synthesis. Administration of proteasome inhibitors induced a dose-dependent impairment of protein synthesis, which was evident at nanomolar concentrations, using two different proteasome inhibitors. The impairment of protein synthesis was observed to be fully reversible within the first 6 h of inhibitor treatment. There was an apparent relationship between the levels of inhibitor necessary to impair protein synthesis and reversibility of impaired protein synthesis, with subsequent development of neuron death. Together, these data suggest that there appears to be an interplay between the proteasome and protein synthesis in neurons, where short-term declines in protein synthesis are beneficial and long-term inhibition of protein synthesis potentially contributes to the toxicity of proteasome inhibition.

## MATERIALS AND METHODS

### Materials

Proteasome inhibitors were obtained from Calbiochem (San Diego, CA, USA); all cell culture supplies were obtained from GIBCO Life Sciences (Gaithersburg, MD, USA). The rabbit reticulocyte lysate was purchased from Promega (Madison, WI, USA). Radioactive isotopes were purchased from Perkin-Elmer (Boston, MA, USA). The columns for RNA purification were purchased from Ambion (Austin, TX, USA). All other items were obtained from Sigma Chemical (St. Louis, MO, USA).

### Neuronal cultures

Neuron cultures were established as described previously (23,24). Briefly, the cerebral cortex of 18-day-old Sprague-Dawley rat embryos were dissected and manually dissociated in Hanks' balanced salt solution. Cells were plated onto polyethylenimine-coated 35 mm dishes containing neurobasal medium supplemented with B-27 and maintained in a CO<sub>2</sub> incubator at 37°C. Seven days after initial plating, cultures were used for experimentation.

### Neuronal viability

Neuron survival was determined by quantification of neuronal morphology and nuclear morphology as described previously (23, 24). Briefly, viable neurons were counted in premarked microscope fields ( $\times 10$  objective) before experimental treatment and 24 h after treatment, with the viability of neurons assessed by morphological criteria. Neurons with intact neurites of uniform diameter and a soma with a smooth appearance were considered viable. Neurons with fragmented neurites and a vacuolated and/or swollen soma were considered nonviable. The number of neurons with fragmented nuclei (nonviable) was also determined and expressed as the percentage of total number of neurons (23, 24).

### Polyribosome purification and protein synthesis methodologies

Polyribosome enriched fractions were purified and analyzed for their ability to translate proteins as described in previous studies from our laboratories and by others (22, 25, 26).

Briefly, cells were collected in buffer A (320 mM sucrose, 50 mM HEPES, 140 mM potassium acetate, 4 mM magnesium acetate, 2.5 mM dithiothreitol, pH 7.5), using a 2.5 vol for tissue of buffer A. The cell lysates were homogenized and centrifuged to generate a polyribosome fraction to be used for *in vitro* protein synthesis assays. The polyribosome content was determined by optical density<sub>280</sub> (22, 26). To measure protein synthesis, equal amounts of polyribosomes were placed in buffer B (10 mM potassium acetate, 0.1 mM magnesium acetate, 0.5  $\mu$ M ATP, 0.5  $\mu$ M GTP, 0.5  $\mu$ M creatine phosphate) containing 50  $\mu$ g/ml creatine phosphokinase. The total volume of the reaction was brought to 99  $\mu$ l, and 1  $\mu$ l of <sup>35</sup>S methionine (10  $\mu$ Ci) was added. To directly measure the functional activity of ribosome, the cell lysate was centrifuged at 1500 *g* and the supernatant was treated with nuclease. Purified luciferase mRNA was then added for *in vitro* translation. For mRNA translation experiments, total RNA was purified from treated cells and translated into protein using a commercially available rabbit reticulocyte lysate system (nuclease-treated; Promega, Madison, WI, USA). After incubation at 37°C for 90 min, the proteins were precipitated overnight at 4°C using 250  $\mu$ l ice-cold 25% trichloroacetic acid containing 2 mg/ml methionine. The solution was then centrifuged at 13,100 *g* for 10 min at 4°C, washed twice with 10% TCA, and the resulting incorporation of <sup>35</sup>S methionine into protein was measured using a scintillation counter. For analysis of protein synthesis *in vivo*, cultures were incubated with <sup>35</sup>S methionine (10  $\mu$ Ci) for 30 min (in methionine-free medium), proteins were precipitated using TCA, then counted using scintillation counter.

### RNA synthesis

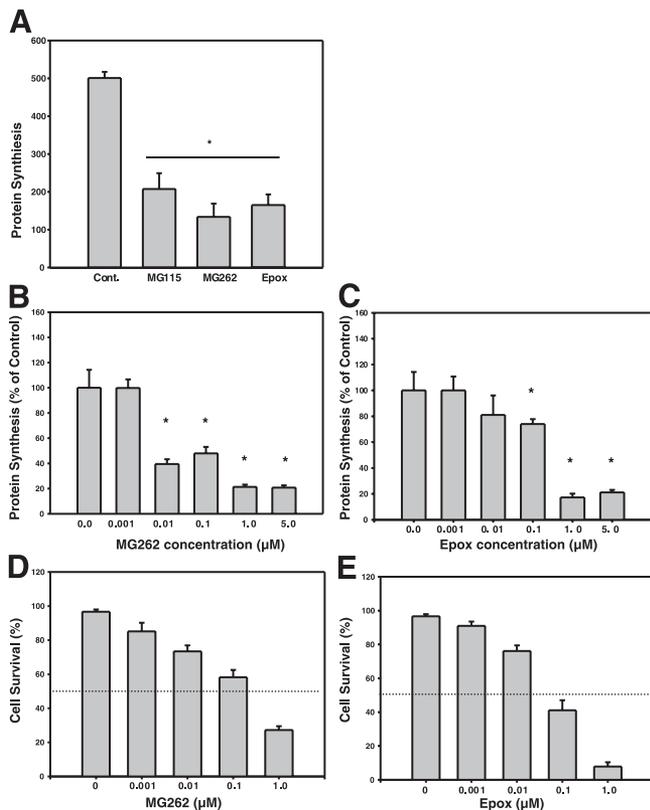
For analysis of RNA synthesis *in vivo*, cultures were incubated with <sup>32</sup>P uridine triphosphate (10  $\mu$ Ci) for 30 min. Total RNA was isolated from treated neurons according to the manufacturer's instruction (TriReagent<sup>®</sup>, Sigma). The mRNA was separated by running total RNA through oligo-(T) affinity column (binding and washing buffer: 0.5 M NaCl, 10 mM TrisHCl, 0.5% SDS, 0.1 mM EDTA, pH7.5; elution buffer; 10 mM TrisHCl, 1.0 mM EDTA, pH7.5; Ambion). Total RNA, eluted mRNA, and the resulting rRNA were precipitated with ethanol and measured using a scintillation counter.

### Statistical analysis

For all determinations of statistical significance, a Student's *t* test was used, with *P* values below 0.05 considered statistically significant.

## RESULTS

To elucidate the potential relationship between proteasome inhibition and alterations in protein synthesis, we conducted studies in which primary rat cortical neurons were treated (1  $\mu$ M) for 3 h with multiple different inhibitors of the proteasome (MG115, MG262, epoxomicin). This concentration and time point were selected based on the fact that for each inhibitor there is a significant inhibition (>40%) of proteasome function in neuronal cultures (data not shown). After treatment with proteasome inhibitors, we observed that all three inhibitors significantly inhibited neuronal protein synthesis (**Fig. 1A**). We then conducted experimentation to elucidate a dose-response profile for



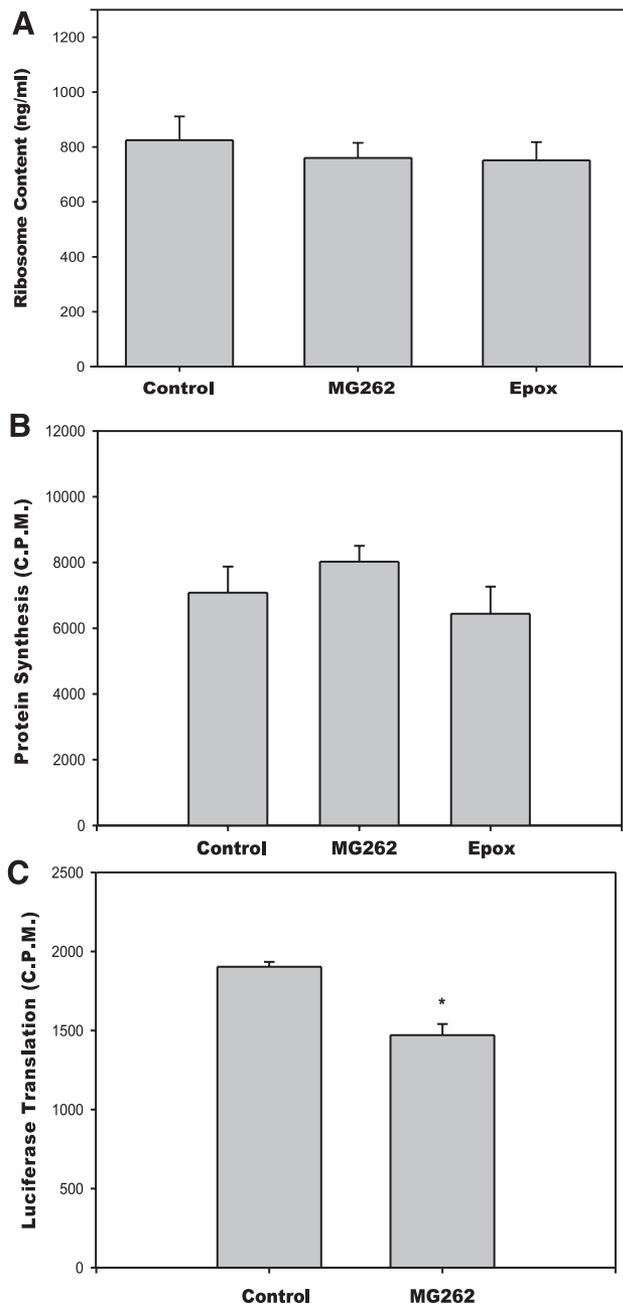
**Figure 1.** Proteasome inhibitors impair protein synthesis and induce neuron death. *A*) Administration of 1  $\mu\text{M}$  different proteasome inhibitors (MG115, MG262, epoxomycin) for 3 h dramatically inhibits the protein synthesis in primary rat neurons. After 3 h administration, the dose-dependent effects of proteasome inhibitors on neuronal protein synthesis was measured in MG262 (*B*) and epoxomycin (*C*)-treated neuronal cultures. After 24 h administration the dose-dependent effects of proteasome inhibitors on neuronal viability was measured in MG262 (*D*) and epoxomycin (*E*)-treated neuronal cultures. Neuron viability was determined using repeated counts of premarked fields as outlined in Materials and Methods. Data are the mean and SE of 3 independent experiments. \* $P < 0.01$ .

MG262- and epoxomycin-induced impairments in neuronal protein synthesis. Concentrations of MG262 as low as 10 nM (Fig. 1*B*) induced significant impairments in neuronal protein synthesis. In contrast, epoxomycin did not significantly inhibit neuronal protein synthesis at 10 nM concentrations but did significantly inhibit protein synthesis at concentrations  $\geq 100$  nM (Fig. 1*C*). For each inhibitor, these concentrations were also observed to significantly inhibit multiple peptidase activities of the proteasome (J. N. Keller and E. Dimayuga, unpublished observation) and are well below the micromolar concentrations of proteasome inhibitor used in most neuronal studies. To elucidate the relationship between these early declines in protein synthesis (observed at 3 h) and neuron death, we conducted studies to determine the LD<sub>50</sub> of MG262 and epoxomycin. While no neuron death was observed during the first 12 h of treatment (data not shown), at 24 h the apparent LD<sub>50</sub> of MG262 and epoxomycin was ob-

served to be  $\sim 150$  nM (Fig. 1*D*) and  $\sim 80$  nM (Fig. 1*E*), respectively. These data indicate that the ability of proteasome inhibitors to impair neuronal protein synthesis occurs prior to neuron death, at concentrations that also inhibit proteasome activity, and demonstrate a relationship between the concentrations of inhibitor necessary to impair protein synthesis and the LD<sub>50</sub> for the different inhibitors. Similar results were obtained for MG115 (data not shown).

In the next set of experiment, we sought to elucidate the basis for observed impairments in protein synthesis. In this first set we focused on the possibility that proteasome inhibitor treatment impaired protein synthesis by decreasing the amount of polyribosomes present in neurons, and thereby promoting a loss of protein synthesis. Analysis of polyribosome content revealed there was no significant alteration in polyribosome content at the time points and conditions associated with impaired protein synthesis (Fig. 2*A*). To confirm that the impairment observed in protein synthesis was not due to proteasome inhibitors directly impairing ribosome function, we conducted studies using purified neuronal polyribosomes. In these studies the naive polyribosomes were analyzed for protein synthesis in the presence or absence of proteasome inhibitors. Administration of proteasome inhibitors (1  $\mu\text{M}$ ) directly to neuronal polyribosomes did not induce impairments in protein synthesis (Fig. 2*B*), suggesting that the observed impairments in protein synthesis were not artifacts of proteasome inhibitors directly impairing polyribosome function. We then sought to determine whether the polyribosomes from neurons treated with proteasome inhibitors exhibited any alterations in their ability to synthesize proteins. In contrast to our studies in which naive polyribosomes were directly exposed to proteasome inhibitors, we observed that polyribosomes from proteasome inhibitor-treated cells exhibited a marked decrease in their ability to synthesize proteins (Fig. 2*C*). These data suggest that alterations in ribosome function could be a contributing factor to proteasome inhibitor-induced impairments in protein synthesis.

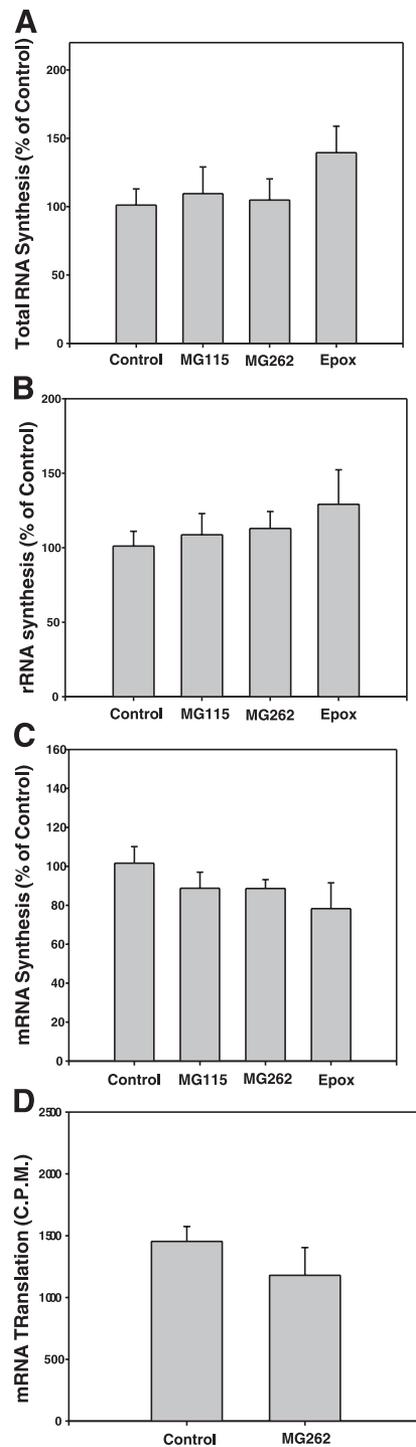
We then sought to elucidate whether the impairments seen in protein synthesis could potentially be mediated by upstream alterations in RNA synthesis. Surprisingly, we observed that none of the proteasome inhibitors used (MG115, MG262, epoxomycin) had any significant effect on total RNA synthesis (Fig. 3*A*). While the total rRNA synthesis gave a trend for being elevated after inhibitor treatment (Fig. 3*B*) and the total mRNA synthesis gave a trend for being decreased after inhibitor treatment (Fig. 3*C*), neither achieved statistical significance. We then sought to elucidate whether the mRNA present after proteasome inhibitor treatment exhibited any alteration in its ability to be translated. These studies revealed that mRNA from neurons treated with proteasome inhibitor did not exhibit a significant alteration in their ability to be translated into protein (Fig. 3*D*), using an artificial protein expression system. Together, these data suggest



**Figure 2.** Proteasome inhibitors impair ribosome function. *A*) Polyribosome content is not altered after 3 h treatment with 1  $\mu$ M MG262 or epoxomycin. *B*) Administration of 1  $\mu$ M MG262 or epoxomycin directly to purified polyribosomes does not significantly alter their ability to synthesize proteins. *C*) Polyribosomes from control (saline-treated) and MG262 (1  $\mu$ M)-treated neurons exhibit an impairment in their ability to synthesize luciferase mRNA into protein. Data are the mean and SE of 3 independent experiments. \* $P < 0.05$ .

that the impairments in protein synthesis after proteasome inhibitor treatment do not appear to be due to gross alterations in RNA synthesis or to gross alterations in the ability of mRNA to be translated.

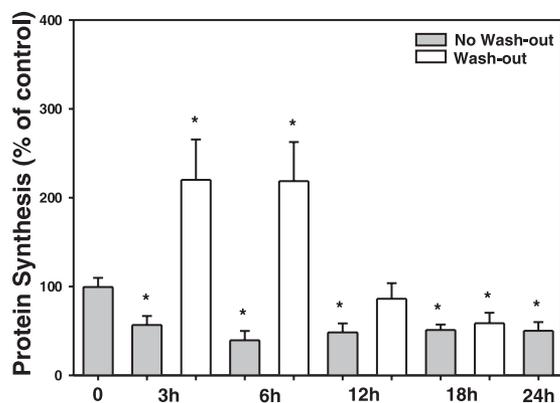
We then conducted experimentation to elucidate whether proteasome inhibitor administration induced a sustained impairment of protein synthesis and to elucidate whether this inhibition was reversible. To



**Figure 3.** Proteasome inhibitors do not alter gross levels or RNA synthesis or mRNA translation. After 3 h administration (1  $\mu$ M) of different proteasome inhibitors, cells were labeled with  $^{32}$ P-uridine triphosphate. The total RNA synthesis was measured in ethanol precipitated cultures, while the amount of mRNA synthesis was determined using an oligo-(T) affinity column, and rRNA synthesis determined as the  $^{32}$ P-uridine triphosphate labeled RNA that was not bound to the column. Values for total RNA (*A*), rRNA (*B*), and mRNA (*C*) are presented as % control. *D*) The amount of TCA precipitate (protein) synthesized from the mRNA of control and MG262 (1  $\mu$ M, 3 h)-treated neurons was not significantly different. Data are the mean and SE of 3 independent experiments.

address the issue of reversibility, we took advantage of the fact that MG262 is a reversible inhibitor of the proteasome (8, 9), with removal of MG262 from the medium resulting in a rapid recovery of proteasome function (data not shown). In these studies, neuronal cultures were treated with the proteasome inhibitor MG262 for increasing periods (3, 6, 12, 18, 24 h) and analyzed for protein synthesis. At each time point examined, significant declines in protein synthesis (Fig. 4), not significantly different from the inhibition observed after 3 h proteasome inhibitor treatment, were observed. We then conducted washout studies to determine whether the ability of proteasome inhibitors to inhibit protein synthesis was reversible. In this experiment neuronal cultures were treated with MG262 for increasing durations (3, 6, 12, 18 h), followed by a washout period. In each study, protein synthesis was analyzed 24 h after the initial administration of MG262, with only the periods of inhibitor treatment and washout variable at each time point. After washout, both the 3 and 6 h MG262 treatments exhibited a pronounced elevation in protein synthesis (Fig. 4). The levels of protein synthesis were significantly elevated even when compared to vehicle-treated cultures (Fig. 4). In both 12 and 18 h MG262 treatments there was no elevation in protein synthesis after washout (Fig. 4). Similar results were obtained using the proteasome inhibitor MG115 (data not shown).

We then conducted experimentation in order to begin to elucidate the neurochemical basis for the reversibility of impaired neuronal protein synthesis. We first sought to elucidate whether there was a reversible impairment of polyribosome function in neurons treated with proteasome inhibitors. In these studies we purified polyribosomes from control (vehicle-treated)



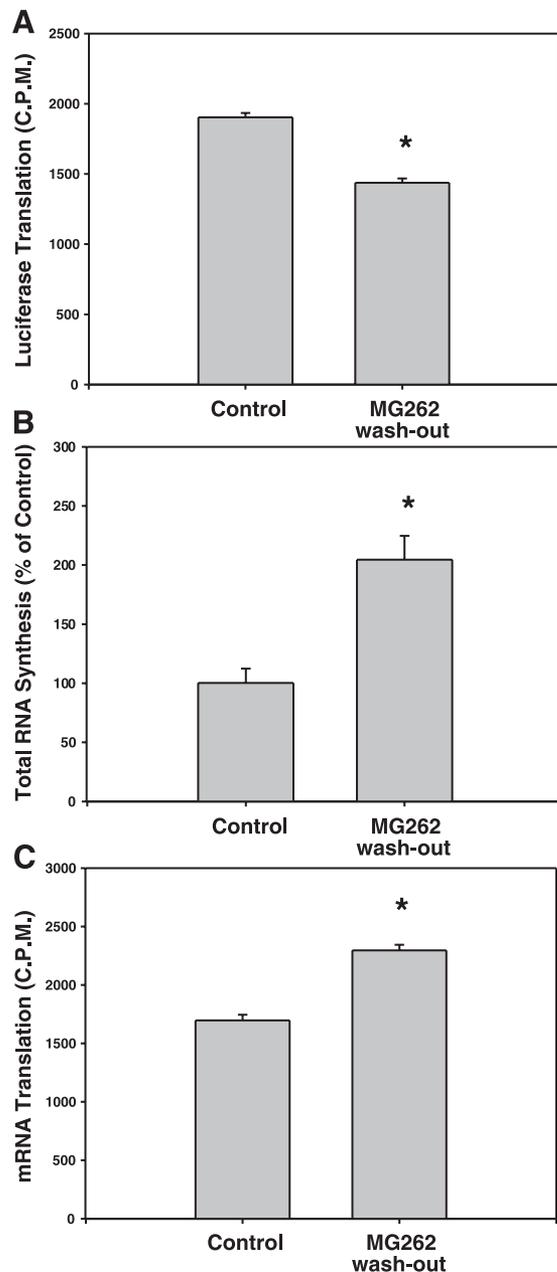
**Figure 4.** The inhibition of protein synthesis is reversible. After treatment with MG262 (1  $\mu$ M) neurons were analyzed for protein synthesis at 3, 6, 12, 18, and 24 h after the addition of proteasome inhibitor (no washout). Additional cultures in each experiment received proteasome inhibitor treatment for 3, 6, 12, or 18 h followed by a washout of the inhibitor (washout). The period of washout for each condition was the difference between inhibitor treatment time, and 24 h (i.e., 3 h inhibitor treatment+21 h washout). Data are presented as the % of control values and represent the mean and SE from 3 independent experiments. \* $P < 0.05$  compared to control cultures.

and cultures that received a 6 h MG262 treatment, followed by a 18 h washout and recovery period. In these studies we observed that even with the removal of MG262 there was a significant impairment in ribosome function (Fig. 5A), suggesting that the recovery of protein synthesis was not due to a recovery of ribosome function. We then examined whether there was any alteration in RNA synthesis during the recovery period, since an elevation in RNA synthesis could potentially stimulate elevations in protein synthesis. In these studies we observed that removal of MG262 significantly elevated RNA synthesis in neuronal cultures (Fig. 5B). We then utilized an *in vitro* protein synthesis system in order to elucidate whether there was any difference in the amount of mRNA translation observed in control (vehicle treated) neuron cultures and neurons exposed to MG262 followed by a washout of the inhibitor. In these studies we observed a significant increase in the amount of translatable mRNA within cultures receiving MG262, followed by washout (Fig. 5C), compared with control cultures. Together, these data suggest that the elevation of RNA synthesis and increased levels of translatable mRNA after removal of MG262 are sufficient to overcome the presence of impairments in ribosome function.

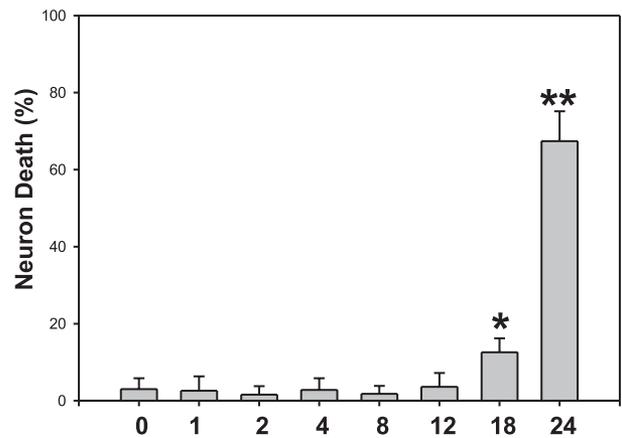
Because we observed a relationship between impairments in protein synthesis and subsequent neuronal death (Fig. 1), we next conducted experimentation to elucidate whether the neurotoxicity of proteasome inhibitor treatment was as reversible as the impairments observed in protein synthesis. In these studies we conducted washout experimentation exactly as outlined for the aforementioned analysis of protein synthesis. In these studies we observed that >12 h of proteasome inhibitor treatment was required for the induction of neuron death (Fig. 6). The neuron death induced by proteasome inhibitor treatment (1  $\mu$ M MG262) was associated with degeneration of neuritic processes and condensation of nuclei, consistent with features of both apoptosis and necrosis (Fig. 7), after 24 h treatment. Cultures receiving the same concentration of inhibitor for a 12 h treatment, or 12 h treatment followed by a 12 h washout, were observed to retain apparently normal neuritic processes and nuclear morphology (Fig. 7). Analysis of neuron death at 48 h revealed that no elevations in neuron death were evident in cultures exposed to proteasome inhibitors for 12 h (data not shown), suggesting that our results were not artifacts of inhibitor washout simply delaying neuron death.

## DISCUSSION

Our data for the first time demonstrate the ability of proteasome inhibitors to decrease protein synthesis in neurons. The effects of proteasome inhibitors were not the result of proteasome inhibitors directly interacting with, and thereby inhibiting, the polyribosome complexes themselves. This is based on the fact that pro-



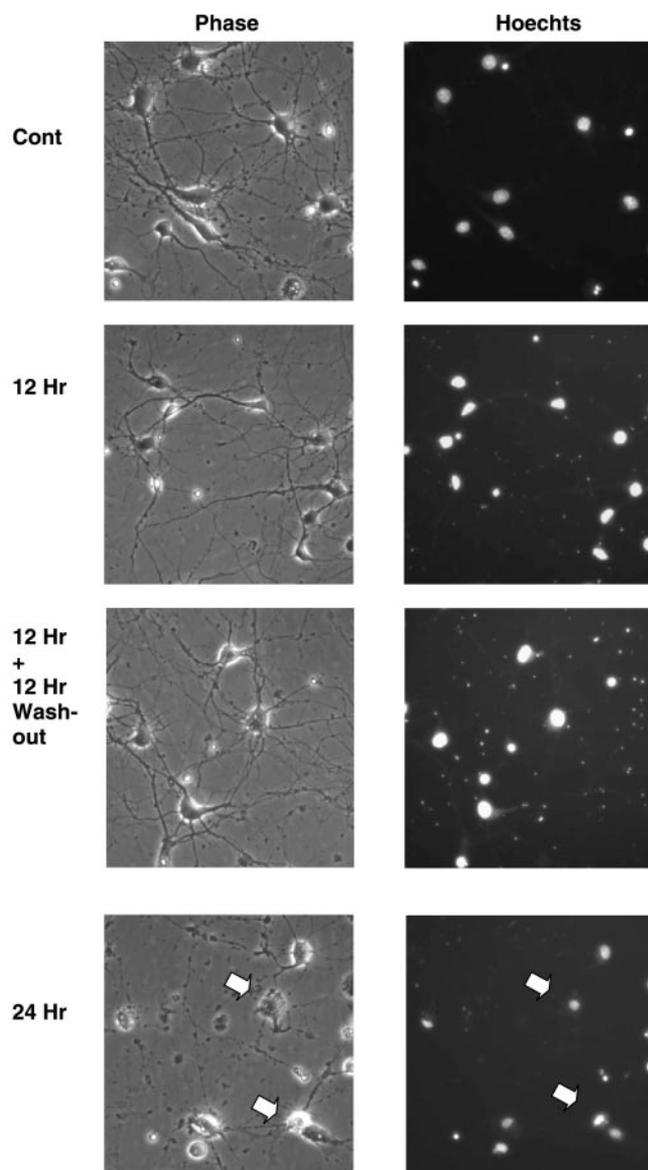
**Figure 5.** The reversibility of impaired protein synthesis is associated with increases in RNA synthesis and translatable mRNA. *A*) Polyribosomes were isolated from control or MG262-washout cultures and analyzed for their ability to synthesize luciferase mRNA into protein. For MG262 washout studies neurons were treated with MG262 (1  $\mu$ M) for 6 h followed by a washout of the inhibitor and a 18 h recovery. These same culture conditions were utilized for analysis of total RNA synthesis after MG262 washout (*B*). In subsequent experimentation the mRNA from control neuron cultures and neuron cultures receiving the aforementioned MG262 washout treatment were analyzed for their ability to be translated into protein (*C*), using an artificial protein expression system. Data are presented as the c.p.m. of TCA precipitate (proteins) or expressed as % of control values. Data represent the mean and SE from 3 independent experiments. \* $P < 0.05$  compared to control cultures.



**Figure 6.** The toxicity of proteasome inhibitors is reversible. After treatment with MG262 (1  $\mu$ M) for 1, 2, 4, 8, 12, 18, or 24 h neurons were analyzed for neuronal viability. Cultures receiving <24 h treatment of MG262 were given a washout of the inhibitor (after the indicated treatment period) and allowed to remain in normal culture medium (free of proteasome inhibitor) for the duration of the experiment (up to 24 h after inhibitor treatment). The period of washout for each condition was the difference between inhibitor treatment time and 24 h (i.e., 1 h inhibitor treatment+23 h washout). Neuron survival was measured using nuclear morphology as an index of neuronal viability. Data are presented as the % of control values and represent the mean and SE from 3 independent experiments. At least 400 cells were counted for each experimental condition. \* $P < 0.05$  compared to control cultures; \*\* $P < 0.05$  compared to control cultures and 18 h cultures.

teasome inhibitors had no effect on the ability of purified polyribosomes to synthesize proteins. Indeed, the effect of proteasome inhibitors on protein synthesis was dose dependent, significantly impairing protein synthesis at nanomolar concentrations. This is well below the concentrations commonly used in neuronal studies, where 1–10  $\mu$ M is a normal treatment range for proteasome inhibitors. At least three different inhibitors, including reversible and irreversible inhibitors, were observed to inhibit protein synthesis, demonstrating that these results are not an artifact of a single proteasome inhibitor. Concentrations of proteasome inhibitor used in the present study significantly impaired protein synthesis, inhibiting both peptidase activities of the proteasome as well as short-lived protein degradation of the proteasome (J. N. Dimayuga and E. Keller, unpublished observations). Taken together, these data strongly suggest that in neuronal cells proteasome inhibition is associated with an inhibition of protein synthesis.

Our findings have important implications for the numerous studies that have utilized proteasome inhibitors to understand the toxicity of proteasome inhibition. For example, studies have used proteasome inhibitors to demonstrate the ability of proteasome inhibition to elevate apoptotic pathways (27–29), induce neuropathology, and induce a variety of neurochemical and neuropathological alterations (10–16). Our data suggest that the effects of proteasome inhibi-

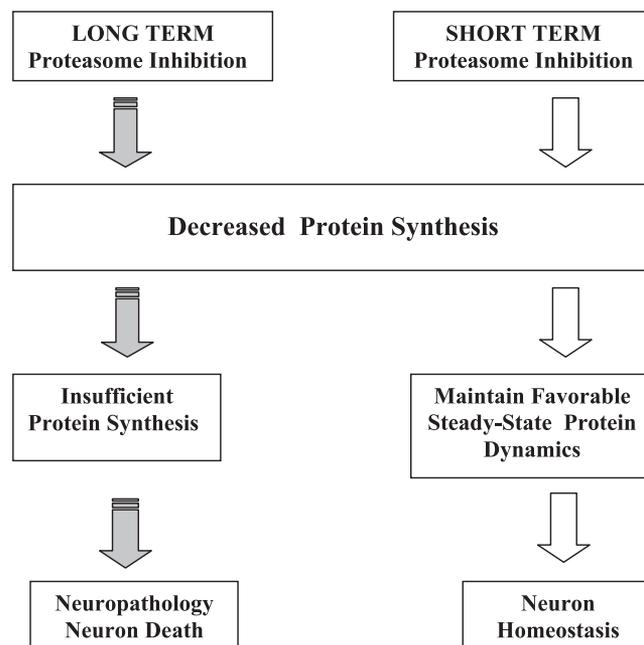


**Figure 7.** Representative images of neurons after proteasome inhibitor treatment. After treatment with MG262 (1  $\mu$ M) neurons were analyzed for neuron survival at 12 h, 12 h + 12 h washout period or 24 h after the addition of proteasome inhibitor. Neuronal viability was assessed using cellular morphology (phase) or nuclear morphology (Hoechst). Cells with fragmented nuclei, shrunken soma, and degenerating neurites are indicated by arrows.

tion on neuronal viability may be mediated in part by subsequent impairments in protein synthesis (Fig. 8). In this model, the short-term impairments in protein synthesis serve a beneficial role in preventing the potentially deleterious accumulation of proteins, and thereby aid in maintaining favorable steady-state protein kinetics in the face of impaired protein turnover (Fig. 8). However, after prolonged proteasome inhibition there is likely to be a pervasive decline in the synthesis of proteins necessary for maintaining homeostasis (Fig. 8), such as proteins involved in regulating heat shock protein response or critical transcription factors (Fig. 8). These prolonged deficits in protein

synthesis mediated in response to proteasome inhibition could then promote neurochemical and neuropathological alterations observed in aging and age-related disease. In particular, declines in protein synthesis could contribute to the protein aggregation observed after proteasome inhibition (due to a potential decline in heat shock protein synthesis) or the induction of apoptosis (due to decreased levels of vital transcription factors). Alternatively, studies in yeast have demonstrated that impairments in protein synthesis promote cell death in a manner that is dependent on ubiquitin depletion (30). In future studies it will be important to elucidate the potential contribution of ubiquitin depletion to the neurotoxicity and declines in protein synthesis observed in the present study.

Previous studies have suggested that declines in heat shock proteins and transcription factors in neurodegenerative diseases are mediated by their being sequestered into protein aggregates. Our data suggest that an alternative explanation for their loss may be declines in protein synthesis, mediated at least in part by proteasome inhibition. This model may explain how in-



**Figure 8.** Model for declines in proteins synthesis serving beneficial effects for short-term proteasome inhibition, and contributing to neurotoxicity with long-term proteasome inhibition. After exposure to a stressor that induces short-term proteasome inhibition, neurons could induce potentially beneficial declines in protein synthesis. This decline in protein synthesis could aid in maintaining favorable steady-state protein dynamics, and prevent the potentially deleterious accumulation of proteins, and thereby contribute to maintaining cellular homeostasis. In contrast, after long-term proteasome inhibition there is likely a prolonged inhibition of protein synthesis that over a prolonged period ultimately leads to insufficient levels of protein synthesis. This deficiency of protein synthesis then contributes to insufficient levels of heat shock proteins, transcription factors, and other cellular components, which ultimately promote the development of neuropathology and neuron death.

creased expression of heat shock proteins and anti-apoptotic proteins exhibit their beneficial effects toward proteasome inhibition (31–33). These data may also help to explain the established differences with regard to the cell type specificity of proteasome inhibitor toxicity, with some cells being extremely resistant to the toxicity of proteasome inhibitors.

In the present study proteasome inhibitors were not observed to significantly alter gross levels of RNA synthesis regardless of the RNA species analyzed. However, our data do not rule out the possibility that the synthesis of individual RNA species are selectively increased or decreased in response to proteasome inhibition. Consistent with this possibility, we noted that individual rRNA species were dramatically elevated in response to proteasome inhibition (supplementary data), although our data cannot exclude the possibility that these individual RNA species are elevated because of increased stability. In a recent study we demonstrated that proteasome inhibition increased RNA oxidation in primary neuron cultures (34), although it was unclear which RNA species were affected. Our present data demonstrate that there is no significant alteration in the ability of mRNA from neurons treated with proteasome inhibitors to be translated into protein using an artificial protein expression system. In future studies it will be important to elucidate whether specific mRNA species have altered synthesis or altered stability in response to proteasome inhibition. We believe that such studies may provide insight as to how proteasome inhibition ultimately affects neuronal homeostasis and potentially contributes to neuronal aging and age-related neurodegeneration. Note that in recent studies we noted that low concentration proteasome inhibition in a neural cell line altered the expression of multiple mRNAs related to Alzheimer's disease, PD, and brain aging (35). Previous studies have demonstrated a role for proteasomes in degrading initiation factors (18,19), which could potentially contribute to alterations in protein synthesis. We conducted extensive analysis of the levels and intracellular localization of eIF2 $\alpha$  and S6 kinase expression after proteasome inhibition and never observed significant alterations in either expression or intracellular localization (data not shown). These data suggest that alterations in initiation factor function does not likely contribute to the observed changes in protein synthesis.

In the present study we observed that the neurotoxicity of proteasome inhibitors was completely reversible within the first 12 h of treatment. This reversibility in toxicity was loosely correlated with the reversible nature of impaired protein synthesis. These data raise the possibility that the time dependence for proteasome inhibitor toxicity may be related to impairments in protein synthesis. While it is possible that cell death pathways are activated soon after proteasome inhibition, our data indicate that a certain amount of activation must be achieved in order to commit the neuron to die. Recent studies have suggested that the early effects of proteasome inhibition on neural cells involves

the induction of pro-survival genes while the later effects of proteasome inhibition include the induction of pro-apoptotic genes (36).

The reversible nature of the toxicity induced by proteasome inhibitors has important implications for understanding the contribution of proteasome inhibition to neurodegenerative conditions. Our data suggest that young healthy cells are able to withstand proteasome inhibition for short periods (<12 h). If the neuron is able to respond to this inhibition by making more proteasomes, inducing a sufficient heat shock protein response, or removing the stressor causing proteasome inhibition, it is likely the neuron will be able to maintain homeostasis. However, if neurons are not able to recover normal levels of proteasome function within a certain time frame, there is likely to be an induction of cell death pathways, and ultimately neuron death. Consistent with the notion of proteasome plasticity, previous studies from our laboratory have demonstrated that in response to oxidative stress, neural cells selectively generate new proteasome subunits that presumably aid in preserving proteasome function and maintaining cellular homeostasis (37). Studies from at least two groups have also demonstrated increased proteasome subunit expression in response to aggregate prone proteins (polyglutamine containing proteins) (38,39), with aggregated proteins capable of inhibiting proteasome function.

Our data indicate that proteasome biology within neurons is complex, and involves the intersection of many different cellular processes, most notably protein synthesis. Incorporating these nuances and subtleties is therefore critical to developing an accurate understanding of proteasomes in brain aging and age-related disorders of the brain. EJ

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