Control of Neuronal Size Homeostasis by Trophic Factor–mediated Coupling of Protein Degradation to Protein Synthesis

James L. Franklin* and Eugene M. Johnson, Jr.‡

*Department of Neurological Surgery, 4640 MSC, University of Wisconsin School of Medicine, Madison, Wisconsin 53706; and ‡Department of Neurology and Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110

Abstract. We demonstrate that NGF couples the rate of degradation of long-lived proteins in sympathetic neurons to the rate of protein synthesis. Inhibiting protein synthesis rate by a specific percentage caused an almost equivalent percentage reduction in the degradation rate of long-lived proteins, indicating nearly 1:1 coupling between the two processes. The rate of degradation of short-lived proteins was unaffected by suppressing protein synthesis. Included in the pool of proteins that had increased half-lives when protein synthesis was inhibited were actin and tubulin. Both of these proteins, which had half-lives of several days, exhibited no degradation over a 3-d period when protein synthesis was completely suppressed. The half-lives of seven other long-lived proteins were quantified and found to increase by 84–225% when protein synthesis was completely blocked.

Degradation–synthesis coupling protected cells from protein loss during periods of decreased synthesis. The rate of protein synthesis greatly decreased and coupling between degradation and synthesis was lost after removal of NGF. Uncoupling resulted in net loss of cellular protein and somatic atrophy. We propose that coupling the rate of protein degradation to that of protein synthesis is a fundamental mechanism by which neurotrophic factors maintain homeostatic control of neuronal size and perhaps growth.

Key words: protein turnover • sympathetic neuron • growth • caspase • nerve growth factor

All proteins turnover at various rates throughout the life of an organism (Goldberg and St. John, 1976; Goldberg and Rock, 1992). This turnover is necessary for replacement of damaged proteins and for alteration of concentrations of particular protein species as required by changing environmental conditions. Most recent studies concerned with protein turnover and its regulation have focused on degradation of individual protein species (Olson and Dice, 1989; Deshaies, 1995; Hochstrasser, 1995; Udvardy, 1996). Whereas regulation of rate of total protein synthesis is a well-established phenomenon with reasonably well-understood molecular mechanisms (Lin et al., 1994; Pause et al., 1994; Schmidt and Schibler, 1995), similar regulation of global protein degradation has been little explored. Recent evidence indicates that most cellular proteins are normally degraded via only a few proteolytic systems (Rock et al., 1994; Ciechanover and Schwartz, 1998). Such commonality in the degradative mechanisms suggests that regulation of these pathways could affect the rate of degradation of many different protein species in concert (Hershko et al., 1971; Hershko and Tomkins, 1971; Epstein et al., 1975; Seglin, 1978; Ballard, 1987; Dice, 1987; Gonen et al., 1994; Rock et al., 1994). This type of regulation is analogous to global control of translation by regulation of the protein synthetic apparatus and could have similar effects on the cellular concentrations of many proteins.

Turnover of total cellular protein has not been examined in any type of neuron. Here we present an investigation of protein degradation in sympathetic neurons and data suggesting coordinate regulation of the degradation of many protein species in these cells. We also demonstrate a previously unknown effect of NGF: to couple the rate of protein degradation to the rate of protein synthesis. This coupling prevents loss of proteins during periods of decreased protein synthesis and, as a result, appears to aid in maintaining homeostatic control of neuronal size.
Materials and Methods

Timed pregnant Sprague Dawley rats were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN). Cell culture medium was purchased from Life Technologies (Gaithersburg, MD) and tissue culture plasticware from Costar Corp. (Cambridge, MA). All chemicals were from Sigma Chemical Co. (St. Louis, MO). Radiolabeled amino acids were purchased from ICN Pharmaceuticals Inc. (Irvine, CA). NanoOrange protein quantitation kits were from Molecular Probes, Inc. (Eugene, OR) and BCA protein assay kits from Bio-Rad Laboratories (Hercules, CA). Electrophoresis reagents and equipment were from Bio-Rad Laboratories. Rat tail collagen was prepared and culture dishes were coated by the method of Johnson and Argiro (1983). Characterization of goat anti-NGF antiserum was done as described by Ruit et al. (1992). Rabbit anti-mouse NGF antibody was from Harlan Sprague Dawley Inc.

Cell Culture

Superior cervical ganglia were dissected from embryonic day 21 rats. Cells were enzymatically and mechanically dissociated from the ganglia and plated on a collage substrate in 24-well tissue culture dishes as described (Johnson and Argiro, 1983; Franklin et al., 1995). In brief, after dissection ganglia were treated at 35°C for 30 min with collagenase (1 mg/ml) followed by 30 min with trypsin (2.5 mg/ml) in Lebovitz’s L-15 medium. The ganglia were placed in culture medium (Eagle medium with Earle’s salts, supplemented with 10% FBS, 100 μg/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 20 μM 2-mercaptoethanol, 1.4 mM L-glutamine, 50 mg/ml 2.5S NGF), tritirated, and then debris separated from dissociated cells by filtration through a size 3–20/14 Nitex filter (Tetko, Kansas City, MO). Approximately 0.5–1 ganglion was plated per well in all experiments. Neuronal cultures were maintained in the same medium used for trituration and plating. Pure non-neuronal cultures (non-neuronal cells in the same medium lacking NGF and containing a polyclonal NGF-neutralizing antibody) were prepared by plating cells in the same medium lacking NGF and containing a polyclonal NGF-neutralizing antibody (either goat or rabbit anti-mouse NGF). No neurons survived this treatment. These cultures contained the same number of non-neuronal cells as did the neuronal cultures. The neuronal cultures were also deprived of NGF by changing culture medium to one lacking NGF and containing a polyclonal NGF-neutralizing antibody. The dilution of each batch of NGF antiserum or antibody needed to block NGF-promoted survival was determined by titration in NGF-containing medium before use in experiments.

Protein Degradation

Metabolic labeling of cellular protein was done 7 d after plating. Unless otherwise stated, cultures were incubated in medium containing 10 μCi/ml TRAN 35S-label (70% l-methionine, 15% l-cysteine) or 10 μCi/ml [3H]leucine. Identical results were obtained with either label. In addition to the labeled amino acids and the amino acids contained in FBS, the labeling medium contained only 10% of the normal amounts of methionine, cysteine, or leucine. Otherwise labeling medium was the same as the standard culture medium. To investigate degradation of short-lived proteins, cultures were exposed to the labeling medium for 1 h, followed by two washes and a 1-h cold chase before the initial time point was taken. To label the short-lived pool separately from the long-lived one was impossible. To enrich for long-lived proteins, neurons were exposed to labeling medium for 24 h. This experiment resulted in a greater percentage of the labeled pool being long-lived because of the continuous turnover of the short-lived pool during the labeling period. The cells were then washed twice with standard culture medium and incubated for 6 h in this medium before the initial time point was taken. Cultures were lysed with a buffer containing 0.5% N-lauryl sarcosine, 1 mM EDTA, and 10 mM Tris HCl, pH 7.2. Protein was precipitated with a solution of ice-cold 10% TCA and retained by filtration through a 0.45-μm nitrocellulose filter. Radioactivity was measured by liquid scintillation counting. Loss of radiolabel is normalized to TCA-precipitable counts measured at the beginning of the experiment. Sister non-neuronal cultures plated (without NGF) at the same time as the neuronal cultures received the same treatments as the neuronal cultures. TCA-precipitable counts in these cultures were then subtracted from the TCA-precipitable counts of the neuronal cultures to correct for incorporation and turnover of radiolabel into non-neuronal cells. The TCA-precipitable counts in the non-neuronal cultures were ~20% of the counts in neuronal cultures. Small variations of this protocol are indicated in figure legends.

TCA precipitation of cellular proteins is the only available technique for investigating degradation or synthesis of total cellular protein and has been used successfully in this capacity for many years (Poole and Wilbo, 1973; Deckwerth and Johnson, 1993; Rock et al., 1994). In addition to proteins, nucleic acids are also precipitated by this method. Because the radiolabeled amino acids used in this study are not incorporated into nucleic acids, most or all of the TCA-precipitated radioactivity reported in this paper is from proteins.

Effect of Inhibitors on Protein Synthesis

For 4-h time points, cells were exposed to the same labeling medium that was used in degradation experiments but containing macromolecular synthesis inhibitors. For 24- and 72-h time points, cells were incubated for 20 or 68 h, respectively, in standard culture medium containing NGF and macromolecular synthesis inhibitors. During the last 4 h of the experiment, cells were exposed to labeling medium containing the inhibitor. This medium was made at the beginning of the experiment and kept in an incubator until use to compensate for any inhibitor degradation that may have occurred. The incorporation of TCA-precipitable counts into proteins was linear over the period of labeling. Appropriate non-neuronal backgrounds were subtracted from each data point in a manner similar to that done in protein degradation studies.

Total Cellular Protein

Total protein content of cultures was determined with NanoOrange Protein Quantitation Kits or by the BCA protein assay. Cultures were washed three times with Eagle medium or PBS, pH 7.2, to remove serum. For NanoOrange determination, cells were lysed with 200 μl of the diluent provided with the kit. Cultures were washed with 200 μl of this solution that had been pooled with the lysates. 50-μl aliquots of the lysate were added to 200 μl of NanoOrange Quantitation reagent and heated at 95°C for 10 min. After cooling to room temperature, fluorescence emission of 200 μl of the treated mixture was measured with a microplate reader by using excitation and emission wavelengths of 485 nm and 590 nm, respectively. For BCA protein assays, cultures were freeze lysed with 200 μl of solution A from the kit. The absorbance of the Cu2+-bicinchoninic acid complex was read with a Bio-Rad 96-well plate reader. Protein concentration was determined from standard curves generated from different concentrations of BSA.

Protein Gels and Autoradiograms

Cultures used for determining degradation of individual protein species were labeled for 24 h as described for studies of the degradation of the total pool of long-lived proteins. However, to increase visualization with autoradiography, 28 μCi/ml TRAN 35S-label was used instead of 10 μCi/ml. Cells were then lysed with 200 μl of 1% Triton X-100 in PBS, pH 7.2, and containing 1 mM EDTA per culture. Equal amounts of the boiled lysates in Laemmli sample buffer were added to each well of 8% Tris-glycine gels (Bio-Rad Laboratories) and proteins were separated by SDS-PAGE. Dried gels of the radiolabeled proteins were then subjected to autoradiography. Autoradiograms were scanned with a Hewlett Packard ScanJet 4c scanner and quantified by SigmaGel (Jandel Scientific, San Rafael, CA).

Curve Fitting, Kinetic, and Statistical Analysis

Protein half-life was determined by the equation \( t_{1/2} = \ln(2)/k \), where \( k \) is the first order rate constant of degradation \( k = (\ln(\%\text{recovered})/t_1 - t_2)/\%\text{recovered} \). The percentage of counts measured at the initial time point remaining after times \( t_1 \) and \( t_2 \) (Doyle and Tweto, 1975). All statistical comparisons were done by unpaired t tests unless otherwise indicated. Curve fitting was done with Sigmaplot (Jandel Scientific). Means in all figures are shown ± SEM.

Results

Effect of Cycloheximide (CHX) on Global Protein Degradation in Sympathetic Neurons

Proteins in rat superior cervical ganglion (SCG) neurons were labeled for 24 h as described for studies of the degradation of the total pool of long-lived proteins. However, to increase visualization with autoradiography, 28 μCi/ml TRAN 35S-label was used instead of 10 μCi/ml. Cells were then lysed with 200 μl of 1% Triton X-100 in PBS, pH 7.2, and containing 1 mM EDTA per culture. Equal amounts of the boiled lysates in Laemmli sample buffer were added to each well of 8% Tris-glycine gels (Bio-Rad Laboratories) and proteins were separated by SDS-PAGE. Dried gels of the radiolabeled proteins were then subjected to autoradiography. Autoradiograms were scanned with a Hewlett Packard ScanJet 4c scanner and quantified by SigmaGel (Jandel Scientific, San Rafael, CA).

Abbreviations used in this paper: ACT D, actinomycin D; ANIS, anisomycin; BAF, Boc-aspararyl (OMe)-fluoromethyl ketone; CHX, cycloheximide; SCG, superior cervical ganglion.
proteins over the period shown in the presence of NGF, but not in its absence. Dotted line is the same as the fitted line in Fig. 1

to distinguish based on the time-course of label decay (Fig. 1

cold-chase protocol, two pools of protein were distin-
guished. When cultures were labeled with a 1-h hot-pulse/1-h cold-chase protocol. (B) Monophasic time-course of loss of TCA-precipitable counts in cultures labeled with a 24-h hot-pulse/6-h cold-chase protocol and then maintained in medium containing or lacking NGF. This pulse-chase procedure resulted in enriched labeling of long-lived proteins. Inset shows TCA-precipitable counts appearing in the medium over 30 h after NGF-deprivation. Open squares in the inset show data from cultures deprived of NGF and maintained in a viable state by 1 \( \mu \text{g/ml} \) CHX. CHX prevented loss of TCA-precipitable counts into the culture medium. The other symbols in the inset have the same meaning as in the main graph. Acid-precipitable counts that disappeared from cells were found in the culture medium as acid-soluble counts suggesting complete degradation of proteins to amino acids and the release of the amino acids from cells (not shown). (C) CHX (1 \( \mu \text{g/ml} \)) did not affect the more rapid component of degradation in the presence or absence of NGF. However, the slower component of degradation was blocked by CHX treatment in the presence, but not absence, of NGF. Dotted line is the same as the fitted line in Fig. 1 A. (D) CHX (1 \( \mu \text{g/ml} \)) almost completely blocked degradation of the long-lived pool of proteins in these cultures over a 72-h period followed first order kinetics with a \( t_{1/2} \) of 60 h. Determination of the time-course of degradation of this protein pool in cultures deprived of NGF over the entire 72 h period was not possible since almost all SCG neurons of the age used here undergo apoptotic death within 48 h of NGF deprivation (Deckwerth and Johnson, 1993). By 18 h after deprivation, TCA-precipitable counts were detected in the culture medium indicating release of undegraded proteins caused by cellular dissolution (Fig. 1 B, inset). This finding is consistent with a previous report of damage to neurons occurring by 18 h after NGF deprivation (Deckwerth and Johnson, 1993). Because of this release, interpretation of degradation data at this and later time points was not possible in NGF-deprived cells. However, the determination of the time-course of degradation of long-lived proteins was possible in neurons deprived of NGF for \( \leq 12 \) h since we found no release of undegraded proteins into the medium had occurred by this time (Fig. 1 B, inset). The time-course of degradation of this pool of proteins in NGF-deprived cultures was the same as that of cultures maintained in NGF for the same 12-h period (Fig. 1 B; \( P > 0.1 \) by paired \( t \) test). The short-lived pool of proteins also showed a similar time-course of degradation during the first 12 h after NGF deprivation in neurons maintained in the presence or absence of NGF (\( P > 0.1 \) by paired \( t \) test;
data not shown). These data demonstrate that no large increase of global proteolysis occurs in the first few hours after NGF deprivation, although such an increase may possibly occur at later times.

To determine whether blocking protein synthesis would influence degradation of the two pools of proteins, cultures were exposed to 1 μg/ml CHX, a concentration shown to block translation completely in these neurons (Martin et al., 1992). This experiment was feasible because, unlike many other types of cells (Wyllie et al., 1980; Koh et al., 1995; Lewis et al., 1995; Miura et al., 1995; Gottlieb et al., 1996), sympathetic neurons can tolerate complete suppression of protein synthesis for long periods and yet remain viable (Martin et al., 1988, 1992). Degradation of the short-lived pool was not affected by treatment with CHX in the presence of NGF (Fig. 1C; P > 0.1 by paired t test). However, in culture medium containing NGF, the more slowly cycling pool showed significantly less decay when CHX was present (P < 0.01 by paired t test).

As with many other types of neurons, the apoptotic death of NGF-deprived SCG neurons is prevented by inhibitors of macromolecular synthesis including CHX (Martin et al., 1988; Scott and Davies, 1990; D’Mello et al., 1993; Garcia-Valenzuela et al., 1995). Indeed, all SCG neurons deprived of NGF can be maintained in a viable state in culture by CHX (1 μg/ml) for 72 h. At the end of this time, CHX can be washed out, NGF replaced, and all neurons will continue to grow and live indefinitely. In contrast to NGF-maintained cultures, the time-course of degradation in NGF-deprived cultures maintained in a viable state by CHX (1 μg/ml) was not significantly different from that occurring in the presence of NGF without CHX (Fig. 1C; P > 0.1 by paired t test). Therefore, in the absence of NGF, CHX did not affect the rate of decay of either protein pool.

Treatment with CHX (1 μg/ml) for 72 h caused almost complete suppression of degradation of the long-lived pool of proteins (Fig. 1D). Again, in contrast to NGF-maintained cultures, the time-course of degradation in NGF-deprived cultures maintained in a viable state by 1 μg/ml CHX was not significantly different from that occurring in the presence of NGF without CHX (P > 0.1 by paired t test; Fig. 1D). The rate of label decay in these neurons displayed first order kinetics with a t1/2 of 55 h. Loss of the effect of CHX on degradation after NGF deprivation was rapid, occurring within 6 h. Therefore, the ability of the cells to reduce degradation of the long-lived pool of proteins after protein synthesis inhibition required the continuous presence of NGF.

**Effect of CHX on Degradation of Individual Protein Species**

To investigate further the relationship between protein synthesis and protein degradation in SCG neurons, we determined the effect of suppressing synthesis with CHX on degradation of individual protein species. Cells were radiolabeled with a 24-h hot-pulse/6-h cold-chase protocol. At various times after labeling cells were lysed, proteins separated by SDS-PAGE, and then amount of label left in protein bands was determined by quantifying autoradiograms of dried gels (Fig. 2A). The time-course of degradation of nine separate protein bands was accurately quantified. As expected, the most heavily labeled proteins were actin and tubulin.

**Figure 2.** Effect of suppressing protein synthesis with CHX (1 μg/ml) on degradation of individual protein species in sympathetic neurons. Autoradiogram showing the time-course of decay of individual proteins. CHX inhibited degradation when NGF was present in the culture medium but not when it was absent. Molecular weight (kD) of protein bands is indicated to the right. Actin decay was completely blocked over a 72-h period when CHX and NGF were both present in the culture medium. In CHX-maintained cultures deprived of NGF, degradation was approximately the same as in control cells maintained in NGF without CHX. (C) Decay of tubulin was also completely blocked by CHX treatment of cultures maintained in medium containing NGF. As with actin, in the absence of NGF decay was approximately the same as in control cells maintained in NGF without CHX. (D) Degradation of a protein(s) with a molecular weight of 100 kD was suppressed, but not completely blocked, by the presence of CHX in NGF-maintained cultures. Again, when NGF was absent, CHX did not affect degradation of this protein(s). We have previously identified the two prominent bands in A as being actin and tubulin (not shown). Each band shown may contain more than one protein. For example, the tubulin band contains both α and β tubulin. n = 3–9 from 2 to 3 separate plottings for each data point. For quantification of degradation, the density of all protein bands was normalized to their densities at T0 on the same autoradiogram.
tubulin (Fig. 2, B and C). The $t_{1/2}$ of actin in neurons maintained in culture medium containing NGF without CHX was 212 h, and that of tubulin was 359 h. In cultures maintained in medium containing NGF, inhibiting protein synthesis with CHX (1 μg/ml) caused complete block of both actin and tubulin degradation over a 72-h period. Rate of degradation of both these proteins in cultures deprived of NGF and maintained in a viable state by this concentration of CHX was not significantly different from that of NGF-maintained cultures without CHX ($P < 0.01$). These results are equivalent to those of Fig. 1 showing block of degradation of long-lived proteins by CHX in the presence, but not in the absence of NGF. All protein bands in autoradiograms of lysates from control cultures were also present in autoradiograms from cultures exposed to CHX, whether or not NGF was present. No new bands appeared when NGF was withdrawn or CHX added, nor was any shift in the molecular weight of any of the bands apparent. Therefore, inhibiting protein synthesis decreased degradation of full-length proteins; proteolytic fragments were not preserved. All proteins had greatly increased half-lives when protein synthesis was suppressed in the presence, but not in the absence of NGF. However, not all proteins exhibited complete suppression of degradation as did actin and tubulin (Fig. 2 D; Table I). These findings further support a role for NGF and protein synthesis in regulating the rate of protein degradation in sympathetic neurons.

CHX and Anisomycin Caused a Rapid, Sustained Clamp of Protein Synthetic Rate: Actinomycin D and NGF Withdrawal Caused a Slower Suppression of Synthesis That Was Not Maintained at a Steady-State Level

To analyze the relationship between effects of inhibitors of macromolecular synthesis on protein synthesis and protein degradation quantitatively, neurons were exposed to different concentrations of inhibitors and effects on both synthesis and degradation assessed. Fig. 3 A shows that the IC$_{50}$ for block of synthesis by CHX was 17 ng/ml with a 4-h treatment and 32 ng/ml with 72 h of exposure. In cells continuously exposed to label and 1 μg/ml CHX for 72 h, no incorporation occurred (not shown) indicating complete, sustained suppression of protein synthesis. Since this concentration of CHX also completely blocked degradation of the long-lived pool of proteins over this period (Fig. 1, C and D), these data demonstrate that reuse of label cannot account for the effect of inhibiting protein synthesis on label loss. Another inhibitor of translation, anisomycin (ANIS), blocked synthesis with an IC$_{50}$ of 8 ng/ml with 4 h of exposure and 22 ng/ml with a 72-h treatment (Fig. 3 B). The dose-response relationship for suppression of synthesis at 72 h was similar to that at 4 h for both CHX and ANIS. Therefore, both CHX and ANIS can be used to “clamp” protein synthesis rapidly at a specific sustained rate. Actinomycin D (ACT D), a transcriptional inhibitor, caused a slowly developing suppression of protein synthesis (Fig. 3 C). Concentrations of ACT D between 1 ng/ml and 300 ng/ml had no effect on protein synthesis over a 4-h period. By 24 h, ACT D caused a dose-dependent suppression of synthesis with an IC$_{50}$ of 7 ng/ml. Suppression was not complete at 24 h. However, after 72 h of treatment, concentrations of ACT D > 10 ng/ml completely blocked protein synthesis (IC$_{50}$ was 4 ng/ml). Presumably, the slow time-course of suppression of protein synthesis by ACT D was a function of the time necessary for mRNA to be cleared from cells before synthesis was affected. Decker and Johnson (1993) reported that NGF deprivation also greatly reduces the rate of protein synthesis in SCG cultures. We repeated this experiment and found that, within 24 h after NGF withdrawal, the rate of protein synthesis had dropped to 18% of control (Fig. 3 D). Each of these treatments, CHX, ANIS, ACT D, and NGF removal, decreases protein synthesis by a different mechanism (Reich and Goldberg, 1964; Wettstein et al., 1964; Grollman et al., 1967): CHX blocks the translocation reaction on ribosomes; ANIS blocks the peptidyl transferase reaction on ribosomes; ACT D binds to DNA and prevents movement of RNA polymerase; and trophic factor withdrawal causes decreased synthesis primarily through changes in complex signaling pathways (Lin et al., 1994). Therefore, these experiments established four independent methods of reducing protein synthesis in SCG neurons.

**Table I. Half-Lives of Individual Proteins**

<table>
<thead>
<tr>
<th>Molecular weight</th>
<th>Identified protein</th>
<th>$t_{1/2}$ (NGF)</th>
<th>$t_{1/2}$ (NGF, CHX)</th>
<th>$t_{1/2}$ (NGF, CHX)</th>
<th>$t_{1/2}$ (NGF, CHX)</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>Actin</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>43</td>
<td>Tubulin</td>
<td>359</td>
<td>237</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>75</td>
<td>75</td>
<td>86</td>
<td>202</td>
<td>76</td>
<td>76</td>
</tr>
<tr>
<td>80</td>
<td>80</td>
<td>81</td>
<td>212</td>
<td>79</td>
<td>79</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>70</td>
<td>129</td>
<td>66</td>
<td>66</td>
</tr>
<tr>
<td>106</td>
<td>106</td>
<td>83</td>
<td>191</td>
<td>74</td>
<td>74</td>
</tr>
<tr>
<td>222</td>
<td>222</td>
<td>112</td>
<td>268</td>
<td>76</td>
<td>76</td>
</tr>
<tr>
<td>241</td>
<td>241</td>
<td>83</td>
<td>212</td>
<td>76</td>
<td>76</td>
</tr>
</tbody>
</table>

Calculated $t_{1/2}$ of individual proteins in sympathetic neurons that received the indicated treatments. The values in parentheses in the fifth and seventh columns indicate percentage change ($\% \Delta$) from $t_{1/2}$ of the same proteins in neurons maintained in culture medium containing NGF but without CHX (third column values). Asterisks indicate that $T2$ values used to calculate $t_{1/2}$ (see Materials and Methods) were significantly different ($P < 0.01$) from the control values used to calculate the $t_{1/2}$ of the third column ($t_{1/2}$). Note that, while the $t_{1/2}$ values in the second to the last column are, in each case, reduced compared to controls, the values of the $T2$ time point used to calculate the $t_{1/2}$ are not significantly different from the controls. ND, no significant degradation. CHX concentration was 1 μg/ml. N = 3–9 from 2 to 3 separate platings for each protein.

CHX, ANIS, and ACT D, but Not NGF Withdrawal, Suppressed Degradation of the Long-Lived Pool of Proteins

Cycloheximide and ANIS both caused a dose-dependent inhibition of protein degradation (Fig. 4, A and B). The dose-response relationship for the block of degradation of long-lived proteins over a 72-h period for either compound was similar to that for inhibition of protein synthesis over this period (Fig. 3, A and B). The IC$_{50}$ for inhibition of degradation were 59 ng/ml for CHX and 54 ng/ml for ANIS. ACT D also blocked degradation, with 1 ng/ml having little effect and 100 ng/ml causing 90% suppression of degradation (Fig. 4 C). Since CHX, ANIS, and ACT D block protein synthesis by three separate means (Reich and Goldberg, 1964; Wettstein et al., 1964; Grollman,
1967), these data strongly suggest that the effect of these inhibitors on degradation was caused by their effect on protein synthesis rather than by non-specific effects on protein degradation. Unlike reduction of protein synthesis by inhibitors in the presence of NGF, suppression of protein synthesis by removal of NGF did not block protein degradation (Fig. 1B). Therefore, while NGF was present, protein synthesis rates were maintained and regulated the rate of degradation of the long-lived pool of proteins; when NGF was removed, protein synthesis declined and this regulation was lost.

Fig. 4D shows that the relationship between the rates of protein synthesis and degradation of the pool of long-lived proteins was linear. The regression line for the relationship between synthesis and degradation in CHX-treated cells has a slope of −1.22 while that for ANIS-treated cells is −0.75. The combined data for CHX and ANIS has a slope of −1.01. These data suggest near 1:1 coupling between rates of protein degradation and protein synthesis. We emphasize that this coupling was not between the absolute amounts of protein produced and degraded but, rather, between relative rates of synthesis and degradation. Since protein synthesis is a process having zero-order kinetics, the rate of synthesis, $P_s$, equals the rate constant of synthesis, $k_s$ (Doyle and Tweto, 1975). Therefore, reducing $P_s$ causes an equivalent percentage reduction of $k_s$. Fig. 4D illustrates that the data presented here denotes that the first-order rate constant of degradation, $k_d$, was a nearly linear function of $k_s$ and, therefore, reducing $k_s$ by a certain percentage reduced $k_d$ by an approximately equivalent percentage.

**Atrophy Preceding Apoptotic Death Probably Results from Uncoupling of Protein Degradation from Protein Synthesis**

One potential physiological role of degradation-synthesis coupling is homeostatic maintenance of cell size. Large cells contain more protein than do smaller cells and the size differences are determined, in part, by the differences in protein concentrations (Rasmussen and Berger, 1982; Clark et al., 1993). In non-growing neurons, total protein content is in a steady-state and, thus, the rate of change of total protein over time, $dP/dt$, is equal to 0. In growing cells $dP/dt > 0$ and in cells undergoing atrophy, $dP/dt < 0$. Our findings show that reducing the rate of protein synthesis, $P_s$, in NGF-maintained SCG neurons decreases the rate of degradation of long-lived proteins, $P_d$, by a proportional amount. This type of coupling should prevent net loss of long-lived cellular proteins when $P_s$ is reduced regardless of the amount of the reduction. Thus, degradation-synthesis coupling may function, at least in part, to maintain neuronal size in the face of constant protein turnover and fluctuating rates of protein synthesis. If so, then uncoupling of the two processes, along with decreased synthesis rate should lead to reduced cellular protein content and atrophic changes. Consistent with this hypothesis, a number of degenerative morphological changes and decreased protein content have been reported in NGF-deprived SCG neurons in the hours preceding commitment to apoptotic death (Deckwerth and Johnson, 1993).

To test whether uncoupling of degradation from synthesis caused somatic atrophy of NGF-deprived cells, we used...
CHX did not influence atrophy.

5-d-old SCG cultures because neurons in cultures of this age do not yet have extensive neurites (Franklin et al., 1995; unpublished observations). Therefore, much of the protein in these cells should be found in somata as opposed to neurites. In the first 24 h after NGF deprivation, we observed profound atrophy of the somas of these cells (Fig. 5, A and B). Average soma diameter of neurons that had been in culture for 5 d was 18.4 ± 0.5 μm (Fig. 5 B). The average diameter of neurons maintained in medium containing NGF for 30 h after this time did not increase significantly (19.04 ± 0.4 μm; P > 0.1). Treatment of neurons maintained in the presence of NGF for this period with CHX (1 μg/ml) did not significantly affect cell diameter (17.8 ± 0.3 μm; P > 0.1). 12 h after NGF withdrawal, soma size was not significantly different from that of control cells (17.8 ± 0.4 μm; P > 0.1). However, during the next 12 h, average soma diameter declined to 13.62 ± 0.5 μm, a highly significant decrease (P < 0.01). Assuming roughly spherical somas, this shrinkage corresponded to a 55% decrease in soma volume. Apoptosis of NGF-deprived SCG neurons is prevented for several days by exposure to 1 μg/ml CHX (Martin et al., 1988; 1992; Deckwerth and Johnson, 1993). However, atrophy was not prevented by maintaining NGF-deprived cells in medium containing CHX (Fig. 5 A). The rates of atrophy of NGF-deprived neurons and deprived neurons maintained in medium containing CHX (1 μg/ml) were not significantly different (P > 0.1 by paired t test). Therefore, although CHX blocked apoptotic death induced by NGF withdrawal, CHX did not influence atrophy.

To determine how inhibiting protein synthesis in the presence or absence of NGF affects total cellular protein and how this, in turn, may be related to atrophy, we determined total protein content in neurons maintained under conditions in which soma diameter was measured (Fig. 5 C). Average protein content more than doubled (in one experiment from 6.3 ± 0.26 μg/culture to 13.54 ± 0.64 μg/culture; P > 0.1) in 5-d-old cultures maintained for 30 h in medium containing NGF. Soma diameter did not increase significantly over this period (Fig. 5, A and B). However, at about this time in culture, neurite outgrowth became significant (not shown). Therefore, this increase in protein (dP/dt > 0) was associated primarily with neurite rather than somatic growth. As shown in Fig. 5 C average protein content of cultures maintained in medium containing NGF and 1 μg/ml CHX for 30 h did not change significantly (dP/dt = 0) compared with that at the beginning of the experiment (6.55 ± 0.8 μg/culture; P > 0.1), again indicating complete suppression of protein synthesis by this concentration of CHX. Degradation of many long-lived proteins should have been almost completely blocked by this treatment. Because cultures deprived of NGF for 30 h contain neurons that have undergone massive cellular dissolution (Deckwerth and Johnson, 1993; Fig. 1 B, inset) the total protein content of such cultures cannot be interpreted within the context of protein loss associated with atrophy. However, in NGF-deprived cultures maintained in a viable state by culture medium containing CHX (1 μg/ml), average total protein content significantly decreased (dP/dt < 0) over the 30-h period (4.18 ± 0.24 μg/culture; P <
of protein synthetic rate by inhibitors of macromolecular synthesis caused an equivalent percentage of reduction in the rate of protein degradation. Removal of NGF caused a profound decrease of protein synthetic rate without a concomitant decrease in rate of protein degradation. Therefore, NGF removal uncoupled degradation from synthesis. Our data also shows that coupling between protein degradation and protein synthesis serves to prevent loss of protein when synthesis rate is reduced and, thereby, appears to protect cells from the atrophic changes that might ensue.

**Relationship of Synthesis–Degradation Coupling to Neuronal Atrophy, Growth, and Apoptosis**

Until recently, regulation of cell size has received little attention (Schmidt and Schibler, 1995; Raff, 1996; Gao and Raff, 1997). There have been no studies directed toward understanding control of size or growth homeostasis in non-cycling cells such as neurons although it seems clear that such regulation must exist. While animal body size is determined by cell number (Raff, 1997), the size of individual animal cells is largely determined by total protein content (Rasmussen and Berger, 1982; Clark et al., 1993). Cytoskeletal proteins form a scaffolding that governs basic cell shape while protein concentration has a profound influence on cell volume. This latter effect results from the role of proteins as the major membrane-impermeant counter anions for the high concentration of intracellular potassium cations (Macknight, 1988). Increased cellular protein content requires increased potassium to neutralize the negative charges on the proteins. To maintain osmotic equilibrium with the extracellular medium, the increase of potassium concentration, in turn, requires an increase of
cellular water. Thus, accumulation of protein is associated with cellular hypertrophy while a decrease is associated with cellular atrophy (Rasmussen and Berger, 1982; Clark et al., 1993).

Often long-lived proteins are structural components of cells or perform “housekeeping” functions while short-lived proteins are transcription factors or rate-limiting enzymes in metabolic pathways (Goldberg and St. John, 1976; Jentsch and Schlenker, 1995). Rapid regulation of the amount of the latter type of protein on a per protein basis is desirable to enable cells to adapt quickly to changing environmental conditions. Structural proteins or housekeeping proteins, particularly in adult neurons, do not need to undergo such rapid up- or downregulation but instead must support the cell relatively unchanged for long periods (as much as a century or more in human and some reptilian neurons). However, during the life of long-lived cells, even these proteins turnover many times. Therefore, it is imperative that protein production and degradation be finely balanced. If such a balance did not exist, small differences in the amounts of protein produced and degraded would be amplified as proteins turnover, eventually affecting cell size and other cellular properties. Thus, the coupling between protein synthesis and degradation described here would seem to be essential for both short- and long-term cellular stability.

The phenomenon that we report here suggests that the relative rates of synthesis and degradation of long-lived proteins in SCG neurons (i.e., the amounts of protein synthesized or degraded per unit time) are nearly 1:1 coupled. For example, when the rate of protein synthesis was reduced by 50% for 72 h, the rate of degradation of long-lived proteins was reduced by ~50% over the same period. We examined the relevance of uncoupling of protein degradation from protein synthesis to the atrophy preceding neuronal apoptosis caused by NGF withdrawal. We found that atrophy may be a direct result of uncoupling of the two processes. When NGF was withdrawn from neurons, protein synthesis declined but protein degradation did not; this caused a decrease in total cellular protein (i.e., dP/dt, 0) and, apparently as a result, somatic atrophy (Fig. 6 A). This is somewhat different from some other types of atrophy that have been investigated where loss in total proteins results primarily from increased degradation rates. For example, in atrophy occurring during cachexia or muscle denervation, protein degradation increases while synthesis may remain unchanged (Furuno et al., 1990; Temparis et al., 1994; Toomey et al., 1995).

A logical consequence of the coupling between protein degradation and synthesis rates is that changing Ps may not significantly alter dP/dt. The rate of degradation, Pd, being first order, is equal to k_d (P_t 1 – P_t) where P_t is the initial protein concentration and P_t is the final concentration measured after a specific time. Since the k_s/k_d ratio for long-lived protein degradation appears to be a constant (C) (Fig. 4 D) and P_s = k_s, the ratio of protein synthetic to degradation rates is given by C/ΔP where ΔP = P_1 – P_2. Therefore, accumulation of the long-lived pool of proteins is entirely controlled by the amount of protein degraded. As a consequence, dP/dt of the long-lived pool of proteins should, theoretically, occur at approximately the same rate regardless of the actual rate of protein synthesis (except of course when P_s approaches 0). To the extent accumulation of long-lived proteins contribute to growth, our data suggest that neuronal growth rate may be buffered from changes in rate of protein synthesis. Therefore, it seems possible that NGF-mediated coupling between protein degradation and synthesis rates could serve not only to prevent atrophy but also to regulate neuronal growth homeostasis. We will address this question in future work.

Apoptosis is a process that, in many cases, requires activation of members of the caspase family of proteases (Stellar, 1995; White, 1996). Once activated, these pro-
teases degrade or activate substrates that are responsible for cell death. Although caspase activation is clearly central to most apoptotic death, including that occurring in NGF-deprived sympathetic neurons (Martinou et al., 1995; Deshmukh et al., 1996; Troy et al., 1996), in SCG neurons this killing activity occurs within the context of cells that have undergone numerous genetic (Estus et al., 1994; Freeman et al., 1994), metabolic, and morphological changes (Deckwerth and Johnson, 1993). Sympathetic neurons deprived of trophic factor exhibit decreased protein synthesis, decreased glucose uptake, neurite fragmentation (Deckwerth and Johnson, 1993), and, as we show here, atrophy well before they are committed to die. A broad-spectrum inhibitor of caspase proteases (Boc-aspartyl(OMe)-fluoromethylketone; BAF) prevents neurite fragmentation and death of NGF-deprived SCG neurons but does not prevent the decrease in protein synthesis or atrophy (Deshmukh et al., 1996). The findings reported here indicate that the reduced protein synthesis and atrophy seen in programmed death induced by trophic factor deprivation, while perhaps necessary for induction or “priming” of apoptosis, are not part of the terminal apoptotic process per se. This idea is further supported by our observation (unpublished) that BAF does not suppress normal protein turnover in NGF-maintained cells and does not appear to have significant effects on proteolysis in NGF-deprived cells maintained in a viable state by the presence of BAF. SCG neurons lose 25% of their total protein content in the hours preceding commitment to die (Deckwerth and Johnson, 1993). Therefore, the large protein loss occurring during apoptotic death is caused by decreased protein synthesis in combination with continuation of a normal rate of protein turnover. The protein substrates degraded by caspase protease that are responsible for cellular demise are, evidently, only a small fraction of total cellular protein. Thus, caspases appear to not be activated until a certain stage of atrophy is reached. Once activated, caspases then cause death within the milieu of a cell that has already undergone massive proteolysis as well as other metabolic, genetic, and morphological changes.

**Possible Mechanisms of Degradation–Synthesis Coupling**

While little is known about how coupling of protein degradation to protein synthesis might occur, we favor the hypothesis of mediation by a protein that positively regulates proteolysis and whose actual or functional half-life is greatly decreased by NGF (Fig. 6B). Such lability would explain why suppression of protein synthesis blocked degradation in the presence of NGF but not in its absence. That is, without NGF, the regulator would have a relatively long half-life and continue to exist at concentrations that enhance proteolysis even in the face of decreased protein synthesis; but, in the presence of NGF, the short actual or functional half-life of the protein would cause its rapid inactivation or removal from the cell when protein synthesis is suppressed. A labile regulator of mRNA degradation has been recently postulated (Ross, 1997) to explain why eukaryotic mRNAs show greatly increased half-lives when protein synthesis is suppressed, a well-known phenomenon (Jacobson and Peltz, 1996) that is strikingly similar to the phenomenon that we report here. Another conceivable, although less parsimonious, mechanism that might mediate coupling is production of a labile, positive regulator of protein degradation by the protein synthetic machinery that is required for degradation in the presence, but not absence, of trophic factor. Such a regulator might be a short-lived protein or a by product of synthesis. Suppression of production of this factor by inhibition of protein synthesis could account for decreased protein degradation.

Global regulation of degradation of long-lived proteins by protein synthesis suggests that proteins in this pool were degraded via the same proteolytic pathway. Evidence suggests that most cellular proteins are degraded during normal turnover by the ubiquitin–proteasome pathway (Rock et al., 1994; Glas et al., 1998). Therefore, this proteolytic system was the most likely site of regulation of protein degradation by protein synthesis. If this were the case, one possible mediator is elongation factor 1α (EF-1α). This protein is required not only for protein synthesis but also for degradation of some proteins by ubiquitin-dependent proteolysis (Gonen et al., 1994). Non-protein mediators of protein degradation include availability of energy (Dice, 1987; Seglin, 1987; Goldberg and Rock, 1992; Deshaies, 1995) and cellular redox state (Halliwell and Gutteridge, 1989). Production of energy was unlikely to be significantly suppressed in neurons exposed to inhibitors of macromolecular synthesis. Large decreases of available energy would influence plasma membrane ion pumps and cause cell swelling by osmotic changes. Because neuronal somas did not enlarge when protein synthesis was inhibited, energy levels were probably not significantly altered in them. Suppressing protein synthesis in sympathetic neurons with inhibitors of macromolecular synthesis causes an increase of cellular glutathione concentration, apparently by shifting cysteine from production of proteins into glutathione synthesis (Ratan et al., 1994). The increased glutathione levels can, in turn, affect cellular redox state. However, increased glutathione levels caused by protein synthesis inhibition occur in the absence, as well in the presence, of NGF in SCG neurons and, therefore, seem an unlikely mediator of protein degradation/synthesis coupling (E.M. Johnson, Jr., unpublished observations).

The question of the generality of synthesis/degradation coupling cannot be easily addressed by the approaches used here because of the inability of most cell types to withstand chronic inhibition of protein synthesis (i.e., inhibitors of macromolecular synthesis are too acutely toxic to examine effects on degradation of long-lived cellular protein). However, a few reports have suggested similar coupling in other systems. For example, accelerated breakdown of long-lived protein caused by serum removal from hepatoma cells in vitro is inhibited by CHX (Hershko and Tomkies, 1971; Epstein et al., 1975). Insulin also inhibits this increased proteolysis. However, the effect of inhibiting macromolecular synthesis on normal protein turnover in these hepatoma cells when exposed to insulin was not investigated. To our knowledge a role for growth factor coupling of protein degradation to protein synthesis has not been investigated in any system. Thus, coupling of degradation and synthesis of long-lived proteins may pos-
sibly be a general function of growth factors acting in many or all cell types.

In conclusion, we have demonstrated that the rate of degradation of long-lived proteins in sympathetic neurons is coupled to the rate of protein synthesis by NGF. This coupling appears to be a means of maintaining global levels of protein concentration in neurons undergoing fluctuations of protein synthetic rate and, as a result, a means for controlling cellular size homeostasis and, perhaps, growth rate.

We thank Dr. P. Lipton and Ms. P. Osborne for critical reviews of the manuscript. We also thank Dr. A.L. Schwartz for reviewing an earlier version of the manuscript; Ms. R. Kirkland for technical assistance; and Dr. D. Creedon for invaluable technical advice.

This work was supported in part by a grant from the Ronald McDonald Foundation, by National Institutes of Health grant NS 24679, and by a grant to the University of Wisconsin Medical School under the Howard Hughes Medical Institute Research Resources Program for Medical grant to the University of Wisconsin Medical School under the Howard Hughes Medical Institute. We also thank Dr. A.L. Schwartz for reviewing an earlier version of the manuscript. We thank Dr. P. Lipton and Ms. P. Osborne for critical reviews of the manuscript. We thank Dr. A.L. Schwartz for reviewing an earlier version of the manuscript.

Received for publication 5 June 1998 and in revised form 30 July 1998.

References


Downloaded from jcb.rupress.org on December 25, 2017


