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Expression of Axonal Protein Degradation Machinery in Sympathetic Neurons Is Regulated by Nerve Growth Factor

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Deficiencies in protein degradation and proteolytic function within neurons are linked to a number of neurodegenerative diseases and developmental disorders. Compartmentalized cultures of peripheral neurons were used to investigate the properties and relative abundance of the proteolytic machinery in the axons and cell bodies of sympathetic and sensory neurons. Immunoblotting of axonal proteins demonstrated that LAMP2, LC3, and PSMA2 were abundant in axons, suggesting that lysosomes, autophagosomes and proteasomes were located in axons. Interestingly, the expression of proteins associated with lysosomes and proteasomes were upregulated selectively in axons by NGF stimulation of the distal axons of sympathetic neurons, suggesting that axonal growth and maintenance requires local protein turnover. The regulation of the abundance of both proteasomes and lysosomes in axons by NGF provides a link between protein degradation and the trophic status of peripheral neurons. Inhibition of proteasomes located in axons resulted in an accumulation of ubiquitinated proteins in these axons. In contrast, lysosome inhibition in axons did not result in an accumulation of ubiquitinated proteins or the transferrin receptor, a transmembrane protein degraded by lysosomes. Interestingly, lysosomes were transported both retrogradely and anterogradely, so it is likely that ubiquitinated proteins that are normally destined for degradation by lysosomes in axons can be transported to the cell bodies for degradation. In summary, proteasomal degradation occurs locally, whereas proteins degraded by lysosomes can most likely either be degraded locally in axons or be transported to cell bodies for degradation.

Key words: proteasome; lysosome; NGF; transport; autophagy

Protein degradation is a process common to all cell types and is important in both cell maintenance and disease (Klionsky and Emr, 2000; Luzio et al., 2007; Tai and Schuman, 2008). Protein degradation is involved in growth cone sprouting and cytoskeletal remodeling (Verma et al., 2005), receptor turnover (Colledge et al., 2003; Arancibia-Carcamo et al., 2009), and elimination of misfolded proteins (Goldberg, 2003). Degradation occurs by three mechanisms: ubiquitin-proteasome degradation, endosomal-lysosomal degradation, and autophagy-lysosome degradation. The ubiquitin-proteasome system degrades cytosolic proteins that have been targeted for proteolysis by the addition of chains of four or more ubiquitin molecules (Thrower et al., 2000). Proteolysis occurs in the proteosome, a protein complex that identifies polyubiquitin chains and cleaves the target protein into small groups of amino acids (Voges et al., 1999). In contrast, monoubiquitination events target proteins to the endosomal-lysosomal degradation system. Transmembrane proteins typically are internalized after addition of monoubiquitin, often on several lysines, and are ultimately degraded by lysosomal enzymes after endosomes fuse with lysosomes (Chau et al., 1989; Hicke, 2001; Luzio et al., 2007). The autophagy-lysosome system functions in large-scale degradation events, resulting in targeted degradation of protein aggregates or entire organelles that have been ubiquitinated and linked to p62/SQSTM1 (Pankiv et al., 2007; Kim et al., 2008; Mizushima et al., 2008; Yang and Klionsky, 2009). Recruitment into autophagic vesicles is mediated by interaction between p62/SQSTM1 and the microtubule-associated protein LC3, a marker for autophagy that is involved in autophagic membrane formation (Kuma et al., 2007; Pankiv et al., 2007).

Defects in protein degradation have been implicated in a variety of neurodegenerative disorders, including Parkinson's disease, Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis (Taylor et al., 2002;

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Rubinsztein, 2006). However, it is not fully understood how defects in protein degradation cause, or develop from, neurodegenerative diseases. Furthermore, the extent to which the protein degradation machinery is localized within axons of large, peripheral neurons is not explicitly known, nor do we know how they contribute to global cellular protein turnover. Although there is evidence for the localization of protein degradation machinery within cell somas and dendrites (Colledge et al., 2003; Arancibia-Carcamo et al., 2009; Lee et al., 2011), it is not clear how this machinery localizes and carries out its functions within axons (Overly et al., 1996; Korhonen and Lindholm, 2004; Song et al., 2008).

We utilized compartmentalized cultures of peripheral neurons to ascertain the abundance and regulation of the proteolytic machinery in axons compared with cell bodies. Protein components of lysosomes, autophagosomes, and proteasomes were all located in axons and lysosomes, and proteasomes were upregulated in response to nerve growth factor (NGF) stimulation. Inhibition of axonal proteasomes resulted in an accumulation of ubiquitinated proteins in axons. However, inhibition of axonal lysosomes did not result in an accumulation of ubiquitinated proteins, suggesting that proteasomal degradation occurs locally, whereas proteins degraded by lysosomes can be transported to cell bodies for degradation.

MATERIALS AND METHODS

Sympathetic Neuron Cultures

Superior cervical ganglion (SCG) neurons were obtained from P0 Sprague-Dawley rats (Charles River, Portage, MI; Goslin and Banker, 1998; Tsui-Pierchala et al., 2002). Neurons were plated on gas–plasma-treated substrates (Harrick Plasma, Ithaca, NY) coated with type I collagen (BD Biosciences, San Jose, CA). Neurons were plated in one of three formats: as mass cultures in the center of 35-mm^2 dishes, as droplets in the center of 22 × 22 mm No. 1.5 coverslips for fluorescence imaging, or as compartmentalized ''Campenot'' chambers (Tyler Research, Edmonton, Alberta, Canada; Tsui-Pierchala and Ginty, 1999; Campenot et al., 2009). SCG neurons were maintained in minimal essential medium (MEM) containing 50 ng/ml NGF, 10% fetal bovine serum (FBS), the antimitotic agents aphidicolin and flourodeoxyuridine (Sigma, St. Louis, MO), and penicillin–streptomycin–glutamine supplement (Invitrogen, Carlsbad, CA). Procedures involving animals were in accordance with the University Committee on Use and Care of Animals (UCUCA) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

NGF Withdrawal and Reintroduction

Sympathetic neurons were maintained for 21 days to allow an NGF-independent, adult phenotype to develop (Goedert et al., 1978; Easton et al., 1997; Orike et al., 2001). To test the effects of NGF on expression of proteins associated with the proteolytic machinery, neurons in compartmentalized cultures were deprived of NGF for 48 hr on both their cell bodies and their axons (see Fig. 3A), a period of time sufficient to extinguish NGF signaling (unpublished observations). After NGF withdrawal, cultures were stimulated either with NGF (50 ng/ml) or with medium alone for 4 or 48 hr. Cellular lysates were then collected from the cell body and terminal compartments.

Monitoring Protein Degradation

Inhibition of protein degradation was achieved by selectively inhibiting proteasomes using 5 μM epoxomicin (Biomol Research Laboratories, Plymouth Meeting, PA), lysosomes using 1 μM concanamycin A (Biomol Research Laboratories), and autophagy using 10 mM 3-methyl-adenine (3-MeA; Sigma). All inhibitors were used at doses similar to those suggested by the manufacturer and in other published reports for inhibition of the various pathways with minimal acute toxicity. After 24 hr of inhibition, lysates were collected separately from the cell body and terminal compartments and examined for the accumulation of ubiquitinated proteins. Mass cultures of SCG neurons were exposed to inhibitors for up to 96 hr to observe their effects on cell viability. Phase-contrast images were collected using a Zeiss Axiovert inverted microscope equipped with a ×10 objective (Zeiss, Jena, Germany).

Immunocytochemisty and Microscopy

SCG cultures were fixed for 10 min in 4% paraformaldehyde buffered with phosphate-buffered saline (PBS), permeabilized for 5 min in 0.1% Triton X-100 in PBS, and blocked for 1 hr in 4% bovine serum albumin (BSA), with shaking. Samples were incubated overnight with a rabbit antiproteasome subunits antibody (1:500 dilution; Invitrogen) on a rotary shaker at 4°C and were then incubated for 2 hr with Alexa-488 goat anti-rabbit secondary antibody (1:1,000 dilution; Invitrogen) with shaking and counterstained for 30 min with Alexa-568 phalloidin (1:200 dilution; Invitrogen). Confocal imaging was performed using an SP5 inverted confocal microscope equipped with a resonance scanner and a ×60 glycerol immersion objective (Leica, Wetzlar, Germany). Lysosomes were labeled using the fluorescent dye LysoTracker Yellow HCK-123 (Invitrogen). Live SCG cultures were incubated for 45 min in culture medium containing LysoTracker and were then imaged in resonance scan mode (16,000 HZ) within a humidified culture chamber maintained at 37°C, 5% CO_2 (TokaiHit, Shizuoka-ken, Japan). Images were collected as single optical sections, with fluorescence and phase-contrast images acquired simultaneously every 2 sec to track lysosome movement. Nocodazole was applied for 30 min after each initial imaging session, and additional images were collected to demonstrate the importance of microtubules for lysosome transport.

Immunoblotting

It was not possible to obtain sufficient amounts of protein to assay the biochemical activity of protein degradation systems in axons. Therefore, immunoblots were used to assess the relative abundance of proteins involved in protein degradation. Protein extracts were loaded onto 4–20% Tris-glycine gradient minigels for SDS-PAGE and were transferred to a

PVDF membrane (Millipore, Billerica, MA). Membranes were rinsed in TBST (10 mM Tris, pH 7.4, 100 mM sodium chloride, 0.1% Tween 20) and blocked for 1 hr in TBST containing 2% BSA. Membranes were then incubated for 3 hr in the primary antibody diluted in 2% BSA-TBST at room temperature or overnight at 4°C. Horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA), diluted in 2% BSA-TBST, were used for detection along with a chemiluminescent substrate (Thermo Scientific, Waltham, MA). The following primary antibodies were used: rabbit anti-PSMA2 (1:1,000 dilution; Cell Signaling, Danvers, MA), rabbit anti-LAMP2 (1:2,000 dilution; Invitrogen), rabbit anti-LC3B (1:1,000 dilution; Cell Signaling), mouse anti-transferrin receptor (1:500 dilution; Invitrogen), rabbit antitotal ubiquitin (1:200 dilution; Sigma), rabbit anti-beclin (1:1,000 dilution; Cell Signaling), rabbit anti-atg5 (1:1,000 dilution; Cell Signaling), goat anti-actin (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-MAP2a/b (1:1,000 dilution; Abcam, Cambridge, MA), rabbit anti-tau (1:1,000 dilution; Cell Signaling), mouse anti-multiubiquitin (1:500 dilution; Stressgen, Victoria, British Columbia, Canada), and mouse anti-p62 (1:1,000 dilution; Abnova, Taipie, Taiwan). Axon growth in compartmental cultures can be highly variable from chamber to chamber, and differences in overall protein levels between experiments often cannot be avoided. Thus, actin immunoblotting was used for protein normalization between compartments. Actin is known to be a good marker for the amount of axons and cell bodies in this culture system, so actin normalization has been adopted by many investigators who perform immunoblotting on compartmentalized cultures. Furthermore, actin accurately reflects the response of neurons to NGF, a neurotrophic factor that increases ATP synthesis, protein synthesis and growth, and indicates the overall increases or decreases in cellular metabolism and axon growth upon manipulation of the trophic status of the neurons.

Image Analysis and Statistical Analyses

Scanned images of X-ray films were imported into ImageJ and processed using the gel analysis tool. Integrated density values obtained from Western analysis were reported as mean values ± SEM. Values were normalized to both actin loading controls reprobed from the same blot and within-blot (vehicle-treated) controls. In some cases, the immunoblot images do not exactly match the quantified data. This is due to inherent variations in the amount of neuronal axons and cell bodies between individual compartmented chambers, reflected in the actin controls. Lysosome movement was measured by using the ImageJ MTrackJ plugin (Erik Meijering, Erasmus MC—University Medical Center, Rotterdam, The Netherlands). Equal numbers of lysosomes moving either retrogradely or anterogradely were tracked for at least three frames, and the average speed was calculated and statistically compared. Student's unpaired *t*-test and one-way ANOVA on ranks were used as tests of statistical significance where appropriate. $P < 0.05$ were considered to be significant. Data analysis was performed in Sigmaplot with Sigmastat (Systat Software, Chicago, IL).

RESULTS

Expression and Localization of Protein Degradation Machinery in Peripheral Neurons

Immunolabeling and immunoblotting with antibodies for MAP2a/b, which is selectively localized in dendrites, and tau, which is specific for axons, indicated that the vast majority of neurites produced by sympathetic neurons in vitro were axons (Fig. 1). Furthermore, MAP2 was absent from the distal axon compartments of compartmentalized cultures, suggesting that only axons extend into the side compartments (Fig. 1C,D).

Lysotracker labeling was used to determine the distribution of lysosomes in the cell bodies, axons, and terminals of mass cultures of SCG neurons (Fig. 2A,C,E). Lysosomes were abundant in the cell bodies of SCG neurons and in large caliber axons extending away from the cell bodies (Fig. 2A) and were also present in the distal axon and terminal regions of SCG neurons (Fig. 2A,C,E). Acidic vesicles (lysosomes, late-stage autophagic vesicles) were observed as either small (<1 μm), single structures or larger (≥1 μm) aggregates. It is possible that larger structures represented autophagic vesicles that had fused with lysosomes.

Immunocytochemical labeling for proteasome subunits revealed that proteasomes were located in both the cell bodies and the distal axons/terminals of SCG neurons (Fig. 2B,D,F). Proteasome labeling was diffuse and evenly distributed throughout the cell, as suggested by the large extent of overlap with phalloidin labeling of the actin cytoskeleton. Proteasome and actin signals did not overlap completely, and proteasomes, for example, were not observed in growth cones (Fig. 2F, arrows).

Immunoblotting of protein extracts collected from mass cultures of SCG neurons and cell bodies and distal axons of compartmentalized sympathetic neurons confirmed the results from Lysotracker labeling and provided additional insights into the localization of protein degradation machinery. Immunoblotting demonstrated that lysosome (LAMP2), proteasome (PSMA2), and autophagesome (LC3, ATG5, and beclin-1) markers were all present in both the distal axons and the cell bodies of SCG neurons (Fig. 2G). LAMP2 was observed as a fully glycosylated species (~110 kDa) and in hypoglycosylated form (<110 kDa). The fully glycosylated (mature) species was enriched in SCG axons, suggesting that lysosomal constituents are posttranslationally modified and assembled in the cell bodies before being transported to the axons in their mature form. Quantification of immunoblots revealed that only LAMP2 showed a significant difference in relative expression level between SCG cell bodies and terminals, but this was due mostly to an absence of hypoglycosylated LAMP2 in distal axons (Fig. 2H). Expression of proteasomes, lysosomes, and autophagesomes was also observed both in the cell bodies and in the distal axons of sensory neurons from the dorsal root ganglion and spinal motor neurons, indicating that these data may be generalized to other neurons that innervate peripheral structures, not just

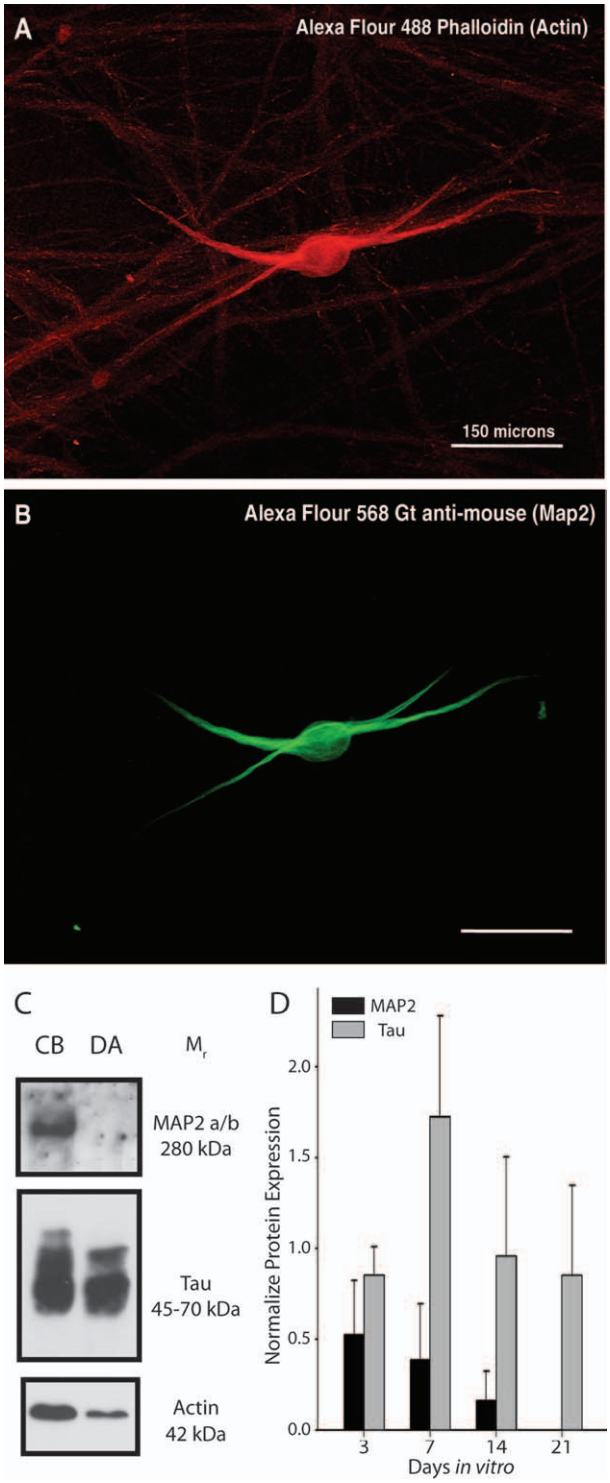

Fig. 1. Axons are present exclusively in the side compartments of compartmentalized cultures. **A,B:** Immunofluorescence revealed that dendrites (MAP2-positive structures) were less abundant than axons and extended only a short distance from somas. **C:** Immunoblotting of lysates from cell bodies and distal axons revealed that MAP2 protein was not present in the distal axon compartment, in contrast to tau, which was highly enriched in distal axons, relative to actin. **D:** The relative abundance of MAP2 protein declined over time in culture.

sympathetic neurons (Fig. 2I). These results indicate that protein degradation pathways are present in the distal axons of neurons, although they are somewhat less abundant than in the cell bodies.

NGF Regulates the Expression of the Proteolytic Machinery

NGF is a target-derived neurotrophic factor that is required for axon growth and target-mediated survival during development as well as maintenance of adult neurons (Sofroniew et al., 2001; Ernsberger, 2009). Because protein degradation facilitates these processes, we hypothesized that NGF-dependent growth and maintenance are linked to NGF-dependent regulation of the proteolytic machinery. After 48 hr of NGF withdrawal, NGF was reintroduced to the distal axons of sympathetic neurons, and protein extracts were collected either 4 or 48 hr later from both the cell bodies and the distal axons (Fig. 3A,B). Immunoblots were performed using protein extracts collected from neurons given medium alone, stimulated with NGF for 4 hr, or stimulated with NGF for 48 hr (Fig. 3). The expression levels of both LAMP2 and PSMA2 increased in the distal axons after NGF stimulation. LAMP2 expression was significantly increased in the distal axons compared with the cell bodies after 48 hr of NGF treatment (Fig. 3C). After 48 hr of NGF stimulation, LAMP2 expression was also significantly higher in the distal axons than after 4 hr of NGF stimulation. PSMA2 expression was not significantly different after 4 hr of stimulation but increased significantly in the distal axons after 48 hr NGF application (Fig. 3D). PSMA2 and LAMP2 increased by more than fourfold compared with distal axons treated with medium alone. In contrast, no significant differences in LC3 expression were observed among any of the conditions (Fig. 3E). LC3 was observed in two isoforms in SCG neurons; the larger, 16-kDa isoform represents the senescent LC3-I form and the smaller, 14-kDa isoform represents the cleaved (active) LC3-II isoform (Klionsky et al., 2008). LC3 cleavage indicates membrane fusion events that cause the formation of a fully functional autophagic vesicle. Although no significant differences in LC3 expression were observed upon NGF stimulation, the ratio of LC3-II/LC3-I suggested that autophagy occurred more frequently in cell bodies than in the distal axons of SCG neurons (data not shown). In summary, these results suggest that NGF regulates the axonal expression of proteins associated with lysosomes and proteasomes but does not regulate proteins required for autophagy.

Differential Sensitivity of Sympathetic Neurons to Inhibition of Degradation Pathways

Because NGF, a potent growth and survival factor for SCG neurons, upregulated the expression of proteins required for proteasomal and lysosomal degradation, we investigated how sensitive these neurons would be to inhibition of these protein degradation pathways. Neurons

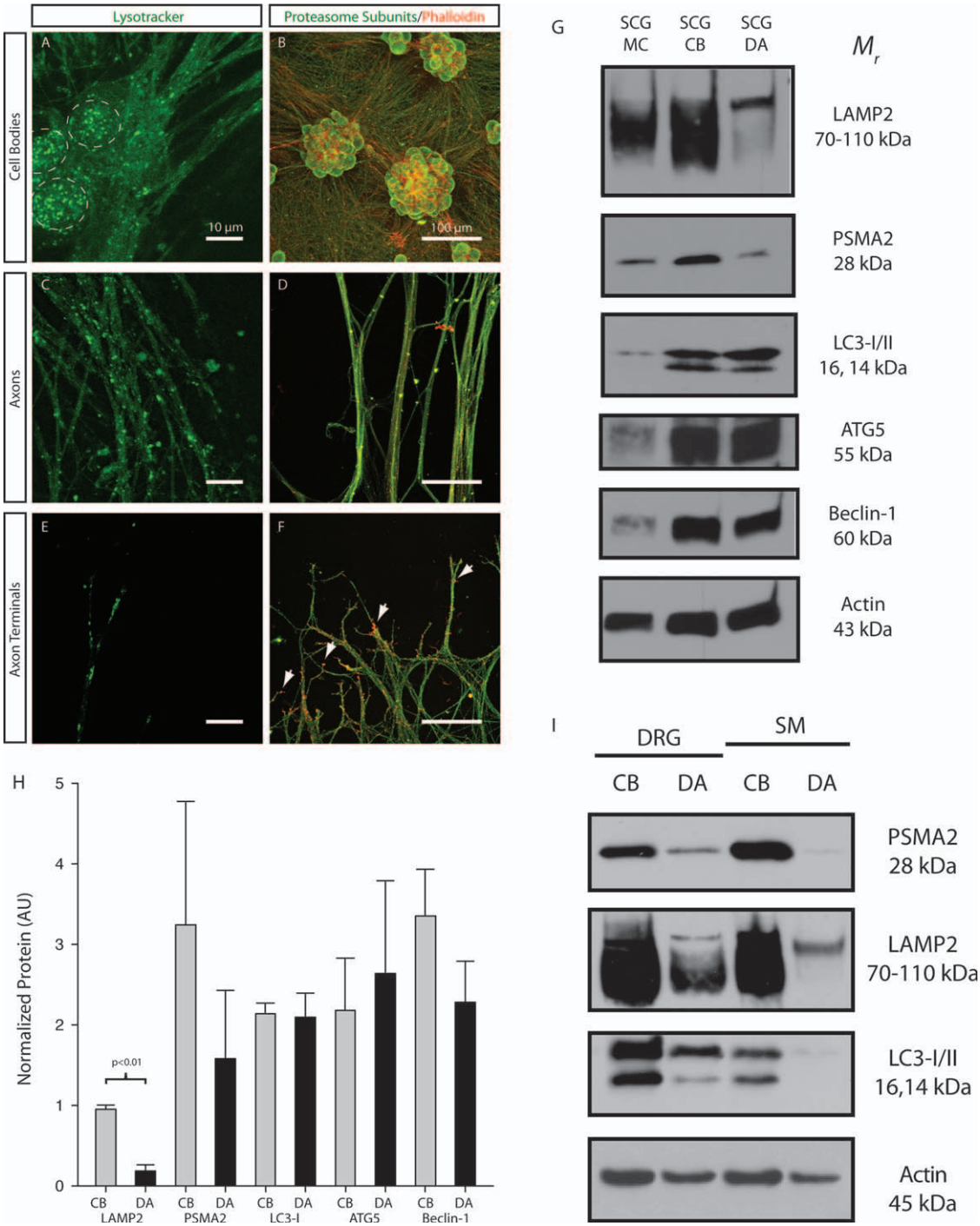

Fig. 2. Lysosomes, proteasomes, and autophagesomes are expressed in both the cell bodies and the axons of peripheral neurons. Lysotracker was used to identify lysosomes (green punctuate structures) in the cell bodies (**A**; encircled), the axons (**C**), and the terminals/growth cones (**E**) of SCG neurons. Immunofluorescence was used to determine the localization of proteasomes (green) in the cell bodies (**B**), the axons (**D**), and the terminals/growth cones (**F**). Phalloidin was used as a counterstain to label F-actin (red; arrows). **G:** Immunoblots for protein extracts obtained from SCG mass cultures (SCG MC), compartmented SCG cell bodies (SCG CB), and compartmented SCG distal axons (SCG DA) confirmed that LAMP2, PSMA2, and three markers for autophagic vesicles (LC3, ATG5, and beclin-1) were expressed in both peripheral nerve tissue and all parts of primary sympathetic neurons. **H:** Differences in relative expression level of proteolytic machinery were observed between cell bodies and distal axons ($P < 0.01$ by Student's unpaired *t*-test). **I:** Dorsal root ganglion (DRG) neurons and spinal motor (SM) neurons express PSMA2, LAMP2, and LC3 in both the cell bodies and the distal axons. These data suggest that some results from SCG cultures may be generalized to other neuronal cell types that innervate peripheral targets. Scale bars = 10 μm in A (applies to A,C,E); 100 μm in B (applies to B,D,F).

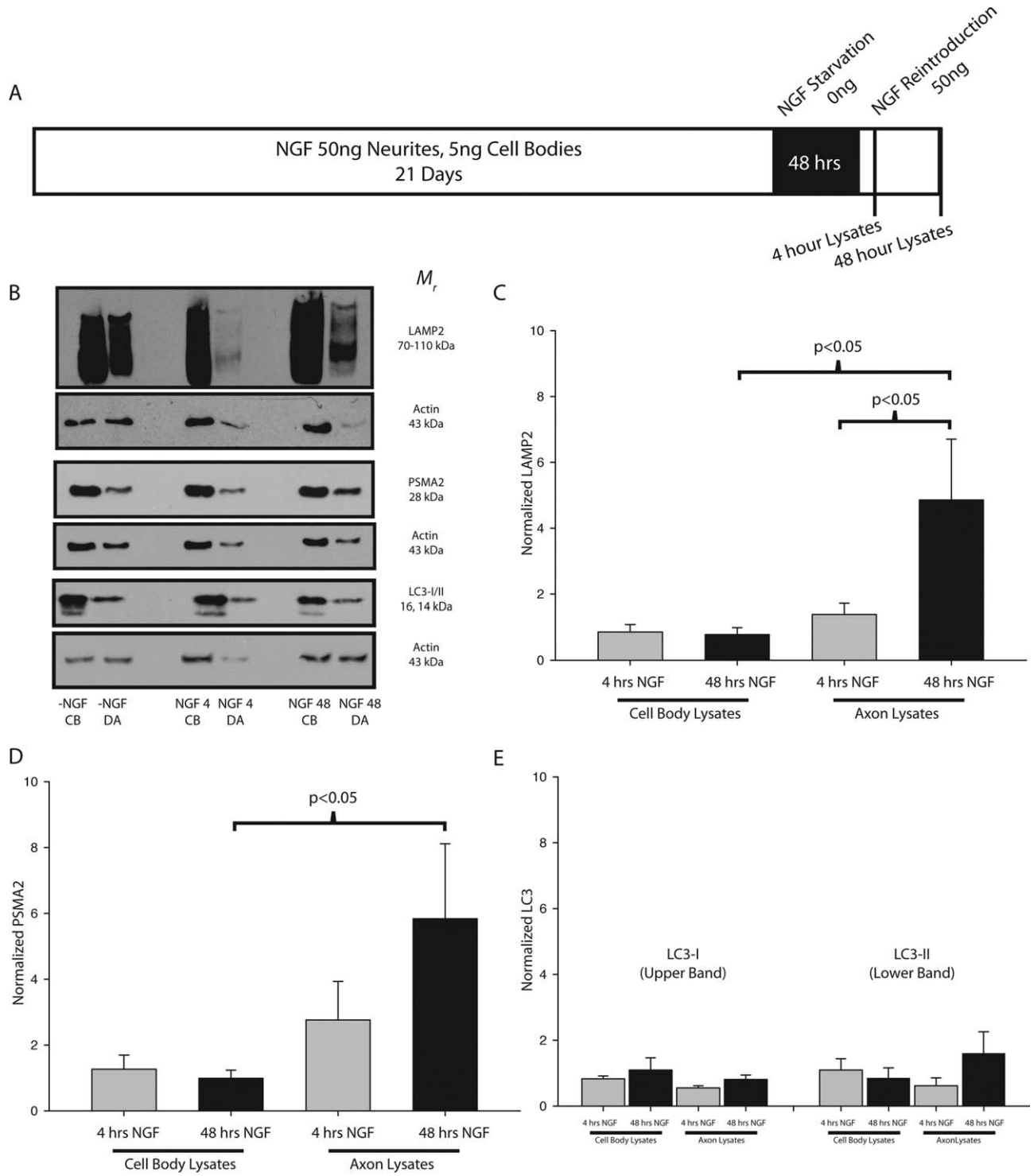

Fig. 3. NGF regulates lysosomes and proteasomes but not autophagosomes. **A:** SCG neurons were cultured for 21 days, deprived of NGF for 48 hr, and then reintroduced to NGF before protein extracts were collected from both the cell bodies (CB) and distal axons (DA). **B:** Representative immunoblots are shown for LAMP2, PSMA2, and LC3 in SCG neurons stimulated on the distal axons with medium without NGF for 4 hr (−NGF CB, DA), 50 ng/ml NGF for 4 hr (NGF 4 CB, DA), and 50 ng/ml NGF for 48 hr (NGF 48 CB, DA). **C–E:** Measurements from NGF-treated samples were normalized to the −NGF controls and actin and statistically compared (one-way ANOVA on ranks). **C:** LAMP2 expression in the distal axons was significantly higher after 48 hr of NGF treatment compared with both the cell bodies at 48 hr and the terminals at 4 hr. **D:** PSMA2 expression increased significantly in the distal axons compared with the cell bodies after 48 hr of NGF treatment. **E:** No significant differences were measured between NGF treatment conditions for LC3. $P < 0.05$ was considered significant.

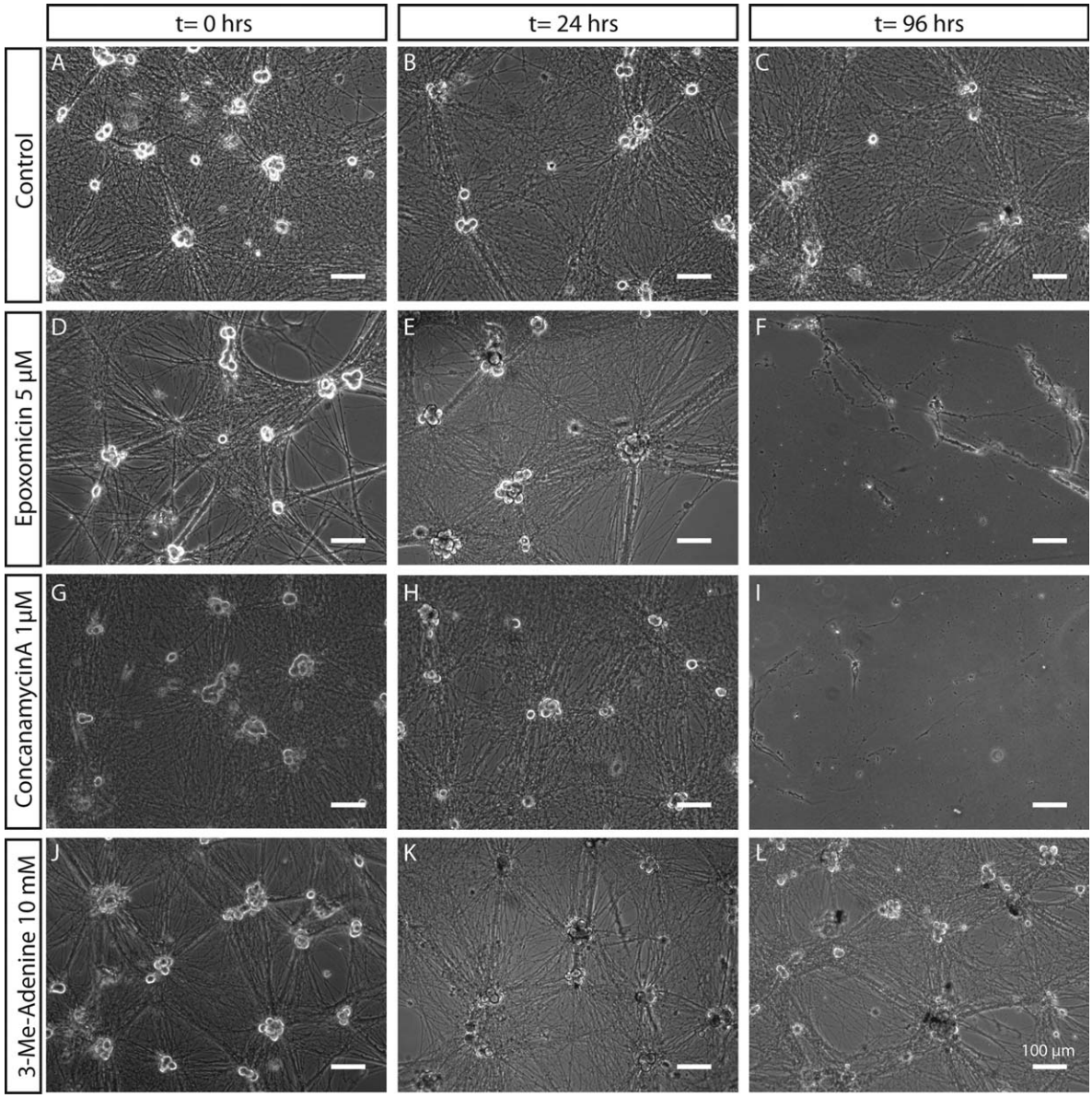

Fig. 4. Differential sensitivity of sympathetic neurons to proteasome, lysosome, and autophagy inhibitors. Mass cultures of SCG neurons treated with vehicle control (**A–C**), epoxomicin (**D–F**), concanamycin A (**G–I**), or 3-methyl-adenine (**J–L**) were observed over the course of 96 hr. SCG morphology was unaffected upon exposure to 3-methyl-adenine for over 96 hr. Application of proteasome or lysosome inhibitors (epoxomicin and concanamycin A respectively), in contrast, resulted in death of SCG neurons over the course of several days, as indicated by the detachment of cell somas and presence of granulated and degenerating neuronal processes. Scale bars = 100 µm.

were exposed to one of three inhibitors of protein degradation, concanamycin, epoxomicin, and 3-MeA, which inhibit lysosomes, proteasomes, and autophagosomes, respectively. The toxic effects of these and other agents are typically obvious in SCG cultures, and are manifested as changes in structure of the cell soma and axonal network.

Sympathetic neurons exposed to the vehicle alone (DMSO) did not undergo degeneration or cell death over 96 hr (Fig. 4). When neurons were exposed to epoxomicin, no acute effects were observed. As expected, the sustained inhibition of proteasomes (96 hr) caused neurite degeneration and cell death, as evidenced by detachment of cell bodies and partial disintegration of processes that remained attached to the substrate (Fig. 4D–F). When neurons were given concanamycin A, cell death also occurred within 96 hr (Fig. 4G–I). However, we observed that neurons were capable of surviving short periods of inhibition (24–48 hr). Inhibition of autophagy using 3-MeA did not result in death of SCG neurons even after 96 hr of exposure (Fig. 4J–L). In fact, no discernible differences in cell morphology were observed between 3-MeA-treated neurons and

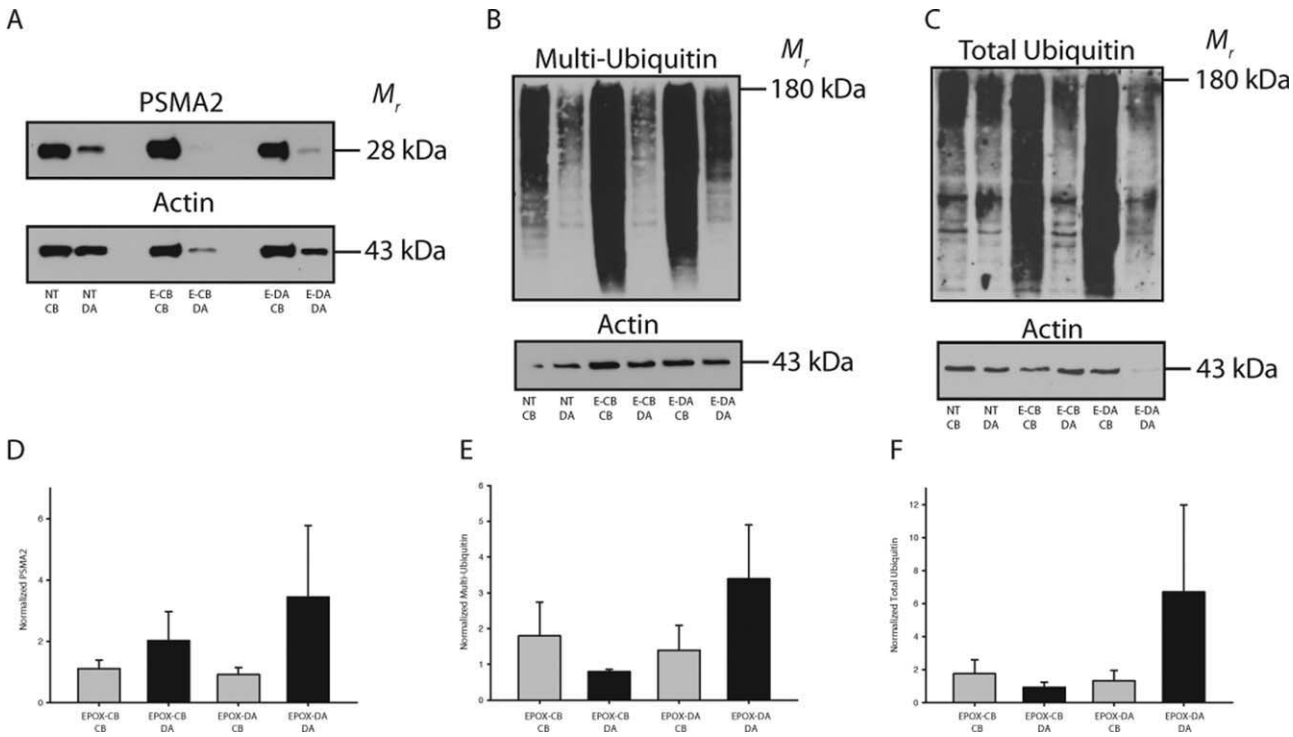

Fig. 5. Localized inhibition of proteasomes results in the accumulation of ubiquitinated proteins in compartmentalized neuronal cultures. **A–C:** Representative Western blots for PSMA2 (proteasomes), multiubiquitin, and total ubiquitin. **D:** Inhibition of proteasome function in distal axons (E-DA) or cell bodies (E-CB) resulted in increases in PSMA2 expression. **E,F:** Ubiquitinated proteins accumulated in the axons/terminals following proteasome inhibition in the terminals. A modest accumulation of ubiquitinated proteins also occurred in the cell bodies after proteasome inhibition in the cell bodies. Values were normalized to actin and an in-blot vehicle-treated control and displayed as mean ± SEM.

neurons given the vehicle alone, suggesting that autophagy was not necessary for the survival of sympathetic neurons maintained in nutrient-rich conditions over a period of 4 days. We chose, based on these observations, treatment time points within the acute (24 hr, low toxicity) period to minimize any toxic effects in subsequent experiments.

Inhibition of Proteasomes in Axons Causes the Local Accumulation of Ubiquitinated Proteins

It has been proposed that one cause of neurodegeneration is the accumulation of toxic or misfolded proteins in the axons of neurons (Taylor et al., 2002). We hypothesized that acute inhibition of the proteolytic pathways in either the cell bodies or the distal axons of SCG neurons would result in the accumulation of ubiquitinated proteins in these compartments. A treatment period of 24 hr was chosen based on the observations that neuronal morphology was unaffected after the acute treatment with either epoxomicin or concanamycin A (Fig. 4E,H).

Representative immunoblots of sympathetic neurons maintained in compartmentalized cultures that were given epoxomicin on either the cell bodies or distal axons are shown in Figure 5A–C. Levels of the proteasome component PSMA2 were measured to determine whether perturbations in protein flux through the proteasome had an effect on the level of proteasome expression (Korolchuk et al., 2010). PSMA2 expression increased in the distal axons in response to proteasome inhibition on the terminals (Fig. 5D). Ubiquitinated proteins increased in the SCG terminals after epoxomicin had been applied to the terminals (Fig. 5E,F). An accumulation of multiubiquitinated proteins destined for the proteasome was also observed in the cell bodies after proteasomal degradation had been selectively inhibited on the cell bodies. Interestingly, similar trends were observed for multiubiquitinated proteins and total ubiquitin (both proteasome and lysosome), which accumulated in axons when proteasomes had been inhibited in axons (Fig. 5F). These data suggest that proteasome inhibition results in the local accumulation of proteins targeted for proteasome degradation and that compensatory mechanisms, such as retrograde transport of ubiquitinated proteins to another cellular compartment, do not aid in degradation after acute proteasome inhibition.

Inhibition of Lysosomal Degradation in Axons Does Not Cause Local Protein Accumulation

We selectively inhibited lysosomes in either the cell bodies or the distal axons of SCG neurons to determine whether deficiencies in lysosomal degradation in axons result in the accumulation of ubiquitinated

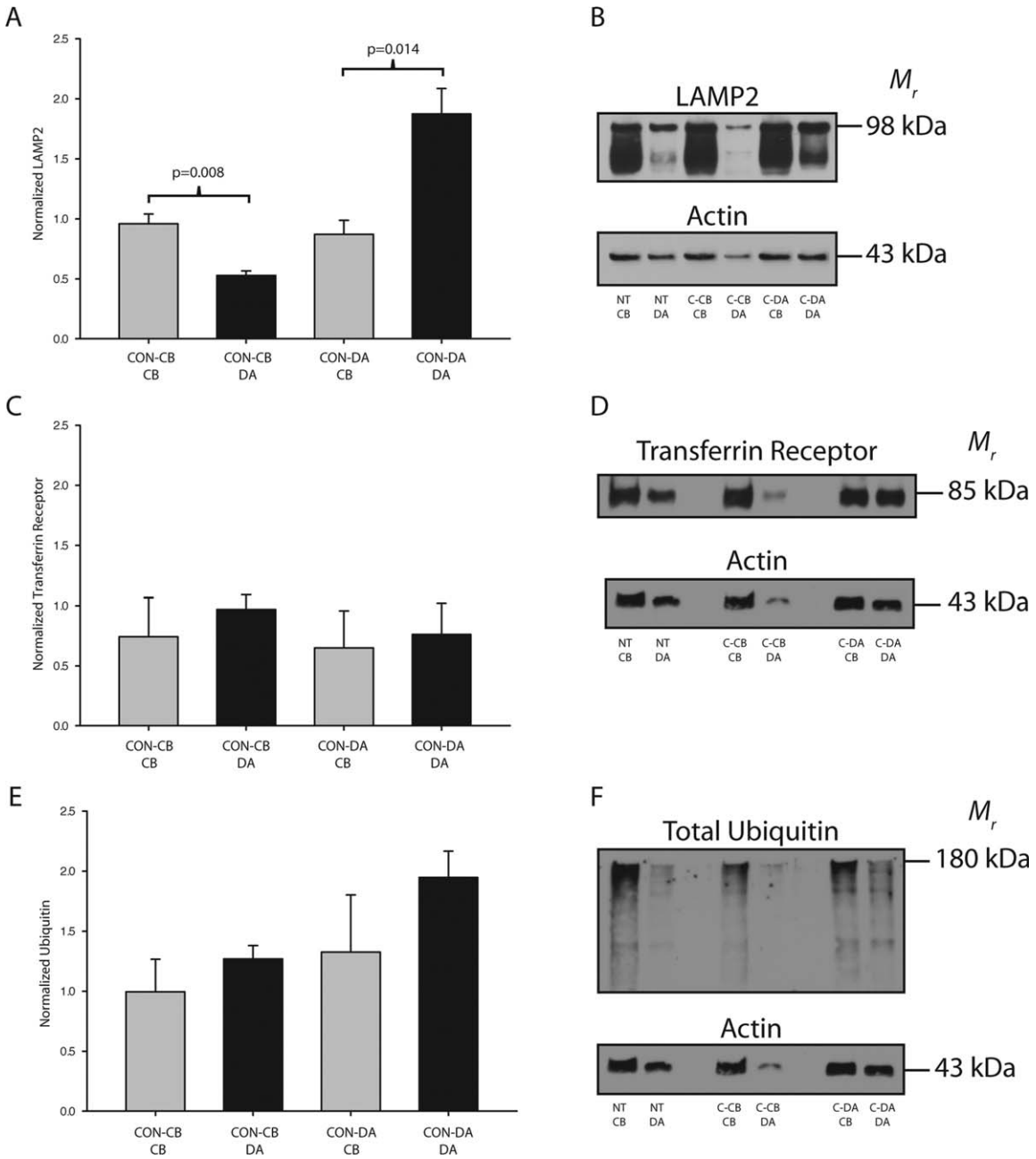

Fig. 6. Selective inhibition of lysosomes does not cause the accumulation of proteins in compartmentalized neuronal cultures. Concanamycin A was used to inhibit lysosomes selectively in either the cell body or the distal axon compartments of SCG cultures. **A,B:** LAMP2 protein expression was greater in the cell bodies after inhibition of lysosomes in the cell body compartment. LAMP2 expression was also significantly greater in the distal axons (DA) after inhibition of lysosomes in the terminal compartment. No significant accumulation of transferrin receptor (**C,D**) or total ubiquitin (**E,F**) was observed following selective lysosome inhibition on either the cell bodies or the distal axons. Bars represent mean ± SEM. $P < 0.05$ was considered significant (one-way ANOVA on ranks). Values were normalized to actin and an in-blot vehicle-treated control.

proteins. LAMP2 expression was significantly higher in the cell bodies of SCG neurons compared with the distal axons when lysosomes were inhibited in the cell body compartment. Likewise, LAMP2 expression was induced significantly in the distal axons compared with the cell bodies when lysosomes were inhibited in the terminal compartments (Fig. 6A,B). These data suggest that either more LAMP2 protein is synthesized or that lysosomes are selectively transported in response to a decrease in lysosomal protein degradation to the cellular compartment where lysosomes are inhibited. Fully glycosylated LAMP2 is enriched in axons, so it is likely that lysoso-

mal constituents are synthesized in the cell bodies and trafficked to the axons after assembly.

In contrast to proteasome inhibition, lysosome inhibition did not result in any trends in the local accumulation of proteins. Transferrin receptor, a model receptor for lysosomal degradation, did not accumulate in response to local lysosome inhibition (Fig. 6C,D). Importantly, no significant differences or trends between experiments were observed for the local accumulation of ubiquitinated proteins, suggesting that lysosome inhibition in subcellular regions of neurons does not lead to the local accumulation of proteins targeted for degradation (Fig. 6E,F). In addition, inhibition of autophagy did not result in local protein accumulation in either axons or cell bodies, as indicated by examination of the expression of the ubuiqitin binding adaptor protein p62 and ubiquitinated proteins (Fig. 7). In summary, the local inhibition of lysosomes does not cause the regional accumulation of proteins targeted for lysosome degradation, such as ubiquitinated proteins and the transferrin receptor.

Lysosomes Are Transported Between Cell Bodies and Axons Via Microtubules

The lack of ubiquitinated protein accumulation following local lysosome inhibition in axons could be linked to the transport of lysosomes within axons. Although lysosomes are capable of moving short distances along actin filaments and microtubules in nonneuronal cells (Cordonnier et al., 2001), it is not known whether lysosomes can be transported over long distances in axons in either a retrograde or a anterograde direction. To examine the possibility that lysosome transport prevented the accumulation of ubiquitinated proteins following lysosome inhibition, we examined the transport properties of lysosomes within axons.

Tracking lysosomes in living sympathetic neurons revealed that lysosomes moved considerable distances along the axons and terminals (Fig. 8A–F). Inspection of overlaid phase-contrast and lysosome images using the fluorescent dye Lysotracker demonstrated that acidic vesicles moved retrogradely from the distal axons of SCG neurons (Griffiths et al., 1988). Two populations of lysosomes were observed in neurons: some lysosomes moved anterogradely toward the terminals, whereas other lysosomes moved retrogradely away from the terminals toward the cell bodies. Lysosomes segregated into two groups based on the direction and speed of their movement (Fig. 8G). Lysosomes moving retrogradely progressed shorter distances over a given time than those moving anterogradely. These data were used to compute the average instantaneous velocity of movement for lysosomes moving anterogradely and for those moving retrogradely. Lysosomes moving anterogradely within axons travelled at an average velocity of 1.2 μm/sec (Fig. 8H). Based on their speed and relatively continuous rate of movement, it is likely that such lysosomes were transported along microtubules by kinesins. The population of lysosomes moving retrogradely travelled at a slower rate (0.46 μm/sec) and moved discontinuously, indicating that they were transported along microtubules by dyneins (Fig. 8H).

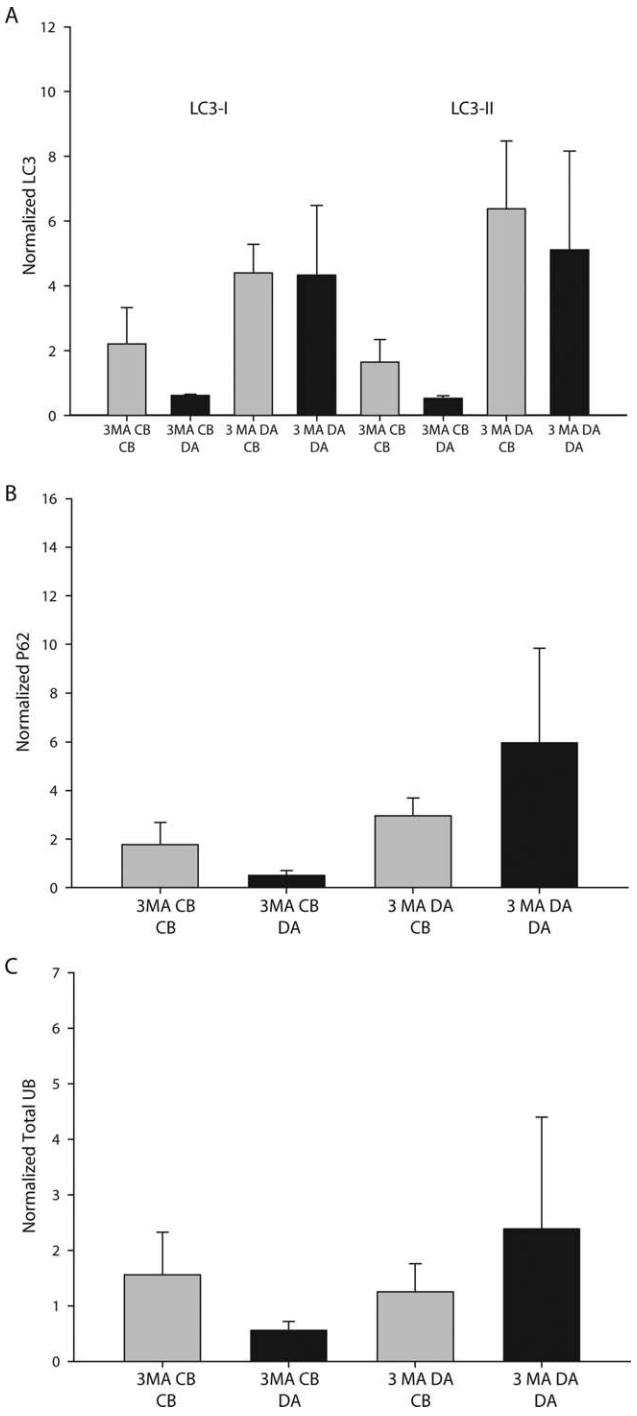

Fig. 7. Inhibition of autophagy does not result in a significant accumulation of degradation products. No significance differences between treatment conditions or trends among experiments were observed for LC3 (**A**), the ubiquitin adaptor protein p62 (**B**), or total ubiquitin (**C**) in response to treatment with the autophagy inhibitor 3-methyl-adenine (3MA). Significance was tested by one-way ANOVA on ranks.

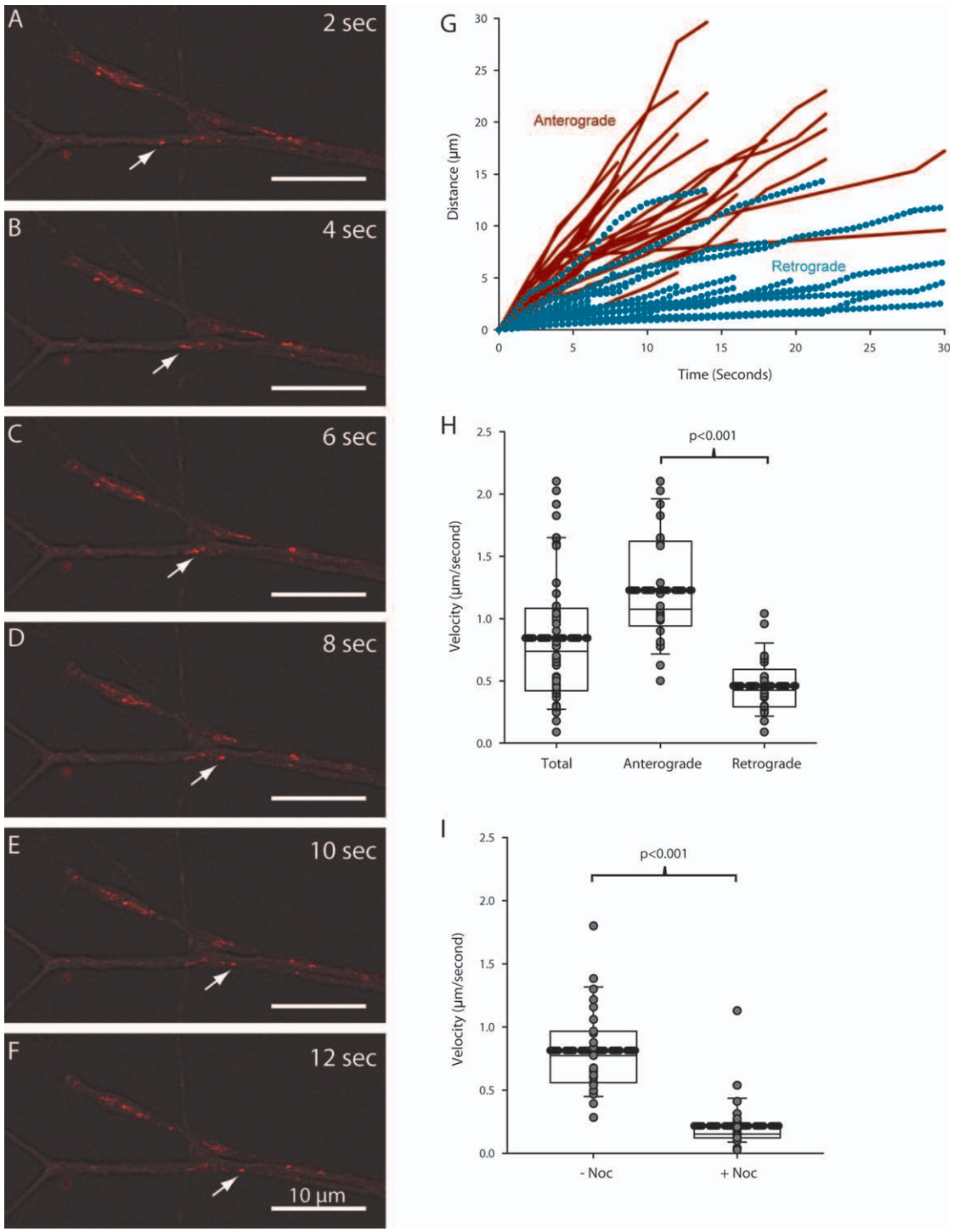

Fig. 8. Lysosomes move retrogradely and anterogradely within the axons of sympathetic neurons. Lysosome movement was tracked in the axons of SCG neurons to assess the impact of lysosome translocation on axonal protein degradation. **A–F:** Lysosomes were observed to move bidirectionally in the terminals of SCG axons. Arrows indicate the retrograde movement of a single lysosome over the course of 12 sec. **G:** Movement profiles for individual lysosomes were color-coded based on the direction of movement (red for anterograde and cyan for retrograde). Lysosomes segregated into two populations based on their movement profiles. **H,I:** Average velocity was plotted for all lysosomes, those moving anterogradely and those moving retrogradely. Average velocity was also measured in the presence of nocodazole. Thick dashed lines represent the mean. Solid lines within the boxes represent the median with first (top and bottom of box) and second (whisker bar) standard deviations. Gray circles represent individual data points. $P < 0.05$ was considered to be significant (Student's unpaired *t*-test). Scale bars = 10 µm.

As expected from these above, lysosome movement was abolished by destabilizing microtubules with nocodozole (Fig. 8I). In the absence of nocodazole, lysosomes moved at an average rate of 0.8 μm/sec. After 30 min of nocodazole treatment, the average velocity of lysosomes was reduced to 0.2 μm/sec. Residual movement was due primarily to Brownian-type motion within the cell, demonstrating that lysosomes depend completely on microtubule transport to move between cell bodies and terminals.

DISCUSSION

Our results indicate that protein degradation is pervasive within the axons of peripheral neurons. Proteins associated with all three protein degradation pathways (proteasomal, lysosomal, and autophagic) were present in both the axons and the cell bodies of sympathetic neurons. These proteins were also present in vitro within the axons of DRG sensory neurons and spinal motor neurons, suggesting that axonal protein degradation is a general property shared among peripheral neurons. The levels of proteins associated with proteasome and lysosome degradation were upregulated in response to NGF treatment, linking the processes of axon growth and maintenance to protein degradation. The inhibition of proteasome activity selectively in axons resulted in local accumulation of ubiquitinated proteins in axons. In contrast, inhibition of lysosome activity in axons did not result in accumulation of either transmembrane receptors typically degraded by lysosomes, such as the transferrin receptor, or ubiquitinated proteins, indicating a potential role of lysosome translocation in protein degradation. Interestingly, retrograde and anterograde lysosome movements were observed in the axons of sympathetic neurons, which were both disrupted upon the destabilization of microtubules with nocodazole. This raises the possibility that subcellular lysosomal inhibition does not cause the accumulation of ubiquitinated proteins because of the transport of lysosomes to other cellular regions.

Localization of Protein Degradation Machinery in Axons

The role of protein degradation in neuronal function and maintenance has been appreciated primarily in the context of protein turnover within cell somas and dendrites (Colledge et al., 2003; Goldberg, 2003; Arancibia-Carcamo et al., 2009). Proteasome degradation has been linked to a number of dynamic processes that occur within dendrites, most notably dendrite morphogenesis, as well as activity-dependent plasticity and long-term potentiation (Ehlers, 2003; DiAntonio and Hicke, 2004). In dendrites, proteasome function is closely linked to synaptic activity. This phenomenon has been illustrated by the recruitment and enrichment of proteasomes into dendritic spines (Bingol and Schuman, 2006). Lysosomal degradation has also been appreciated in the contexts of neurite remodeling as well as turnover of receptor/complexes at postsynaptic structures (Luzio et al., 2007; Song et al., 2008). Alterations in protein degradation by lysosomes and proteasomes have been linked to protein accumulation disorders and degeneration/regeneration of axons (Rubinsztein, 2006). Although it is likely that some protein degradation occurs following retrograde translocation of endosomal vesicles to cell bodies, the abundance of lysosomes, proteasomes, and autophagic proteins within distal axons suggests that local axonal protein degradation contributes to proteome homeostasis within axons. The accumulation of ubiquitinated proteins in axons upon local proteasome inhibition, along with the anterograde and retrograde transport of mature lysosomes, further supports this notion.

NGF and Regulation of Protein Degradation

NGF supports the target-dependent survival, growth, and maintenance of sympathetic and sensory neurons (Sofroniew et al., 2001; Ernsberger, 2009). Our observation that proteasome- and lysosome-associated protein levels were upregulated by NGF selectively in axons suggests that axonal protein turnover is necessary for axonal growth and maintenance. Therefore, a loss of trophic factor support may contribute to degenerative disorders associated with protein accumulation in axons, such as Alzheimer's and Parkinson's diseases, by exacerbating the accumulation of misfolded and/or ubiquitinated proteins (Hennigan et al., 2007). It will be important in future studies to determine the precise function that the NGF-induced increase in protein degradation has in axons, especially because levels of NGF in target tissues often decline after development and during adulthood. Importantly, the increase in proteasomal and lysosomal proteins in axons in response to NGF was greater than the increase in actin, which is a faithful marker of the trophic effects of NGF on global protein levels.

The levels of both PSMA2 and LAMP2 increased selectively in the axons of NGF-stimulated neurons at 48 hr. The levels of LC3 did not increase in axons following NGF stimulation, thus serving as an important control (in addition to actin) that suggests that NGF selectively upregulates proteins associated with proteasomes and lysosomes in axons. It is important to note that autophagy is active in NGF-maintained axons, as evidenced by the appearance of the active form of LC3 (LC3-II) in axonal lysates. This demonstrates that the lack of evidence for NGF regulation of autophagy is not due simply to an absence of active autophagy in these axons. Overall, these data suggest that an augmentation of protein degradation is necessary for some specific aspect of NGF-mediated axon growth or maintenance, rather than simply being necessary as a housekeeping function for axon homeostasis.

Transport Properties of Protein Degradation Machinery

The cell bodies and terminals of most neurons are often separated by large distances, requiring the transport of mRNA, protein components, and complete protein

complexes/organelles for the maintenance of functional axons and terminals. By inhibiting the function of proteolytic machinery within either cell bodies or axons it was possible to understand how transport properties can affect the flux of proteins through degradation machinery. Inhibition of proteasome function resulted in the accumulation of ubiquitinated proteins in the inhibited compartment, suggesting that the ubiquitin-proteasome system (UPS) lacks mechanisms for transport of ubiquitinated proteins out of the inhibited compartment. This supports previous reports indicating that local degradation is particularly important in the UPS (DiAntonio and Hicke, 2004; Bingol and Schuman, 2006). Indeed, proteasomes have been observed to aggregate in regions of high protein turnover. In contrast, protein accumulation was not observed following lysosome inhibition. However, an increase in lysosome-associated protein expression was observed in the inhibited compartments, suggesting either that lysosome expression was upregulated or that lysosome translocation occurred in response to a decrease in local protein degradation. The lack of protein accumulation in concanamycin A-inhibited compartments likely is due to the transport of endosomes or lysosomes out of the inhibited compartment to a region of the neuron where degradation could occur.

Lysosomes were observed to move great distances both anterogradely and retrogradely within neurons, consistent with some previous studies (Overly et al., 1996). These data support a model in which lysosomal cargo can be either degraded in axons or transported between axons and cell bodies for degradation. During intracellular transport, lysosomes can move bidirectionally and undergo fusion events with endosomes and autophagesomes, thus providing a dynamic and locally tuned mechanism for protein degradation to accompany the stable local degradation of the UPS. It is likely that autophagy is also occurring in axons given the presence of fully mature autophagosomes. Even so, some studies have demonstrated that lysosomes function independently of autophagosomes to degrade transmembrane proteins. For example, most plasma membrane-located receptors are trafficked to lysosomes for degradation, and this process does not involve autophagy. Rather, autophagy is involved primarily in the degradation of organelles and large cellular structures, often as a response to nutrient deprivation. The observation that lysosomes can move within neurons to overcome deficiencies in local degradation has implications for neurodegenerative diseases, raising the possibility that diseases affecting axonal transport mechanisms may impede the adaptive ability of the neuron to remove ubiquitinated proteins that are accumulating in axons due to the disease process, thereby impacting the synaptic function of the neuron. It is thus possible that the apparent toxicity of some protein inclusion structures, such as those related to Aβ and tau, is caused in part by deficiencies in the transport of protein degradation machinery within axons.

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