Stage-Dependent Axon Transport of Proteasomes Contributes to Axon Development

Highlights

- Retrogradely moving proteasomes are selectively elevated at the nascent axon
- Controlled proteasome transport establishes asymmetric proteostasis in neurons
- BDNF specifically promotes retrograde proteasomal transport via Ecm29 phosphorylation
- Phosphorylated Ecm29 facilitates proteasome-dynein association

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In Brief

Hsu et al. demonstrate a preferential retrograde axonal transport of proteasomes, establishing the asymmetric proteostasis required for axon formation. This robust, long-range, and temporally controlled proteasome transport is established prior to the formation of the axon initial segment's cytoplasmic transport barrier.
Stage-Dependent Axon Transport of Proteasomes Contributes to Axon Development

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SUMMARY

Axon extension at the growing tip requires elevated local protein supply, with a capability sustainable over long axons in varying environments. The exact mechanisms, however, remain elusive. Here we report that axon-promoting factors elicited a retrograde transport-dependent removal of proteasomes from nascent axon terminals, thereby increasing protein stability at axon tips. Such an effect occurred through phosphorylation of a dynein-interacting proteasome adaptor protein Ecm29. During the transition from immature neurites to nascent axons in cultured hippocampal neurons, live-cell imaging revealed a significant increase of the retrograde axonal transport of fluorescently labeled 20S proteasomes. This retrograde proteasome transport depended on neuron stage and increased with axon lengths. Blockade of retrograde transport caused accumulation of proteasomes, reduction of axon growth, and attenuation of growth-associated Par6 at the axon tip of newly polarized neurons. Our results delineate a regulatory mechanism that controls proteasome abundance via preferential transport required for axon development in newborn neurons.

INTRODUCTION

Axon development starts with a process of symmetry breaking at newborn neurons, where asymmetric accumulation and activation of proteins lead to axon and dendrite specification and neuronal polarization (Cheng and Poo, 2012). Newly specified axons then grow over long distances to reach their synaptic targets. For the initial specification and subsequent growth of axons, it is essential to accumulate specific signaling or structural proteins at the growing axon tip and deplete these proteins elsewhere. Such processes can be achieved by implementing a local positive regulation at the axon tip over other neurites through, for instance, preferential transport or degradation of specific proteins (Cheng et al., 2011a; Namba et al., 2011). While major studies have been focused on preferential transport of proteins such as KIF5/kinesin-1, Par3, TrkB, and NgCAM (Arimura et al., 2009; Burack et al., 2000; Kamal et al., 2000; Nakata and Hirokawa, 2003; Nishimura et al., 2004), less understood are the roles of preferential degradation of proteins in axon initiation and growth.

In eukaryotes, protein degradation is governed by ubiquitin proteasome system (UPS). Proteasome is an enzymatic mega complex composed of at least 33 different subunits, assembled into two parts—a highly conserved, catalytic 20S core particle and one or two 19S regulatory particles. These particles assemble independently with the help of cytoplasmic chaperones before forming the 26S proteasome complex (Coux et al., 1996; Murata et al., 2009). Recent studies indicate that UPS contributes to axon growth and guidance (Campbell and Holt, 2001). In cultured hippocampal neurons, for example, reducing proteasome activity by pharmacological inhibitor MG132 or lactacystin led to the formation of multiple axons (MAs), but the underlying mechanism is unclear (Cheng et al., 2011a; Yan et al., 2006). Likewise, cytoplasmic transport systems in neurites may dynamically regulate proteasome translocations (Bingoi et al., 2010; Djakovic et al., 2012; Hamilton et al., 2012; Hamilton and Zito, 2013), but whether directional proteasome transport could be regulated by extracelluar signals, e.g., axon promoting factors, in a manner that contributes to axon growth or asymmetric protein degradation in polarizing neurons remains unknown.

Here, we examined the spatiotemporal dynamics of proteasomes in unpolarized and polarized neurons. We found an asymmetric transport of 26S proteasomes that occurred early in neuronal polarization and led to selective reduction of protein degradation at the tip of the nascent axon. This process resulted from an enhanced retrograde transport of proteasomes away from the neuritic tip, via microtubule-based, dynein-mediated transport. The axon-promoting factors cAMP and brain-derived neurotrophic factor (BDNF) increased this retrograde proteasome transport at nascent axon without affecting the anterograde direction. Biochemical assays showed an axon-promoting signal promoting PAKA-dependent phosphorylation on the 26S proteasome adaptor extracellular mutant-29 (Ecm29) that enhanced proteasome-dynein interaction. This Ecm29 phosphorylation could be triggered by BDNF in undifferentiated neurites and contributed significantly to axon formation in vitro and neuronal migration in vivo. Further, the degree of preferential transport was stage dependent and became more profound at later stages when cells exhibited extensively long axons. Such
Figure 1. Reduced Proteasome Abundance Correlates with Axon Formation

(A and B) Neurites with high GFPu reporter accumulation (low degradation rate) showed high probability of becoming axons. (A) Images of two example neurons on 1 and 3 DIV, showing accumulation of GFPu in an undifferentiated neurite (marked by asterisk) that later became the axon. The intensity of GFPu is proportional to the brightness of the grayscale image, with high-intensity range marked by the red color. Bar represents 25 μm. (B) Bar graphs summarizing results from all live-imaging experiments as in (A), showing neurites that exhibiting the highest level of GFPu accumulation mostly became the axon. (Top) Neurites were ranked according to the average fluorescence intensity (up to seven neurites) for all cells on 1 DIV (n = 90, from five independent experiments; **p < 0.001, ANOVA with Tukey post hoc test). All GFPu values were normalized by co-transfected volume control tdTomato. (Middle) The average length of neurites, corresponding to those shown in the panel above. (Bottom) The percentage of neurites that differentiated into axons on 3 DIV for the corresponding neurite groups shown in the above panels.

(C) Inverse correlation between proteasome abundance and neurite length. Representative image of a 1 DIV neuron labeled with proteasome marker MV151 (100 μM, 1 hr). Histograms depict the distribution of average length (±SEM, n = 45; white bars) of seven longest neurites (ranked by length for each cell) and average MV151 intensity (±SEM, n = 15; normalized to volume control GFP; red bars) of the neurites corresponding to the neurite groups shown on the right. (D) Images of 3 DIV neurons with MV151 or MV152 labeling (100 μM, 1 hr) for proteasome abundance, immunostained for axon-specific Tau-1 (green), and neuron-specific Tuj-1 (red). The MV151/MV152 fluorescent intensity is proportional to the brightness of the grayscale image. (E) Measured MV151 and MV152 intensity along the axon and dendrites (average of five neurites) of the neuron shown in (D).
phenomena can be explained by a simple mathematical model. Together, these studies delineated a regulatory mechanism for proteasome removal via phosphorylation of the adaptor Ecm29, leading to differential protein stability required for axon development.

RESULTS

Reduced Proteasome Abundance in Nascent Axons In Vitro and In Vivo

To investigate the role of protein degradation in developing neurons, we assessed the status of protein degradation in cultured hippocampal neurons transfected with the short half-life protein degradation reporter GFPu (Bence et al., 2001, 2005; Gilon et al., 1998). Cells were examined 4 hr after plating. In unpolarized neurites (i.e., neurons with multiple neurites of similar lengths) from 1 day in vitro (1 DIV) culture, we found that neurites with the highest GFPu intensity had a higher probability (0.73) of becoming the axon (at 3 DIV) than those with lower GFPu intensity (probability < 0.1; Figures 1A and S1B). The observed GFPu intensity difference was due to protein degradation, as confirmed by transfecting cells with the long half-life (>24 hr) reporter Enhanced Green Fluorescent Protein (EGFP), which exhibited random patterns in unpolarized neurites, without any apparent spatial correlation to their axonal fate (probability <0.3 each; see Figures S1C and S1D).

The asymmetric distribution of GFPu may result from differential proteasome abundance or activity in the neurites. To ascertain whether the asymmetry is due to uneven proteasome abundance, we used fluorophor-coupled activity-based probe BODIPY TMR-Ahx3L3VS (MV151) (Verdoes et al., 2006b). MV151 covalently binds to the catalytic subunit α1, β2, and β5 of proteasomes with a binding capacity of one 20S core harboring at most six MV151 molecules and has been used for live-cell imaging and in-gel fluorescent labeling of functional proteasome complexes (Verdoes et al., 2006a, 2006b) (also see Figures S2A and S2B, which shows that approximately 90% of MV151-labeled puncta were co-transported with ectopic expressed 20S proteasome subunit α4). By incubating cultured hippocampal neurons with MV151 for 1 hr, we observed an asymmetric distribution of MV151 fluorescence intensity (normalized by volume control tdTomato) that inversely correlated with the neurite length in unpolarized, multineurite neurons at 1–2 DIV (Figure 1C). At 3 DIV, we found that neuritic processes with the lowest MV151 fluorescence intensity (equivalent to proteasome abundance) were likely to show highest immunostaining of the axon marker Tau1 (in 42/50 cells; Figures 1D–1F). Thus, we found an early and persistent increase in the retrograde movement of axonal proteasomes at tips of nascent axons (Figures 2A and 2B). In the kymographs of MV151-labeled proteasomes at stage 1, we found nearly 35% of proteasomes exhibiting apparent anterograde (21%) and/or retrograde (12%) motions in neurites, while the rest (67%) remained stationary (Figure S2C). At stage 2, the majority of proteasomes in all neurites became dynamic, and more retrograde than anterograde motions were observed in the longest neurite (the nascent axon). At stage 3, the increase of retrograde movements became more pronounced (~10 events per 180 s; Figures 2B and S2C), as shown by measuring the frequencies of anterograde and retrograde movements that crossed the first 100 μm region of axon (from the soma). Thus, the degree of retrograde flow and hence the differential accumulation of proteasomes between axon and short neurite tips correlate with the stages and increases with the axon length.

In comparison, short neurites showed unchanged frequency and average speed of proteasome movements in either anterograde or retrograde direction throughout stages 1 to 3 (Figures 2B, 2C, and S2C). In addition, the anterograde behaviors of proteasome movements were comparable throughout the three stages (Figures 2B and S2C), suggesting that the differential proteasome abundance between axons and short neurites was mainly due to an enhanced retrograde removal, rather than a lack of anterograde supply of proteasomes in axonal terminals.

To confirm this hypothesis, we disrupted the function and/or distribution of retrograde motor dynein complexes in 1–2 DIV neurons, prior to their axon determination. By both pharmacological and genetic approaches, we found that dynein activities
were not only required for the asymmetric proteasome distribution, but also for axon growth and development. Pharmacologically, treating 2 DIV hippocampal neurons with dynein ATPase inhibitor erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA, 0.5 mM) (Gibbons et al., 1978) or ciliobrevin D (Cilio D, 0.5 mM) for 2 hr led to marked accumulation of endogenous proteasome subunits (e.g., Rpt1 and PA28) at all neurite tips (n = 20–25; Figures 2D, S2D, and S2E). In particular, the accumulation at axonal tips was most prominent (Figure 2D).

Figure 2. Selective Modulation of Retrograde and Anterograde Proteasome Transports in Developing Hippocampal Neurons

(A–C) Preferential increase in the number of retrogradely moving proteasomes in nascent axons. (A) Schematic diagram on developing neurons and kymographs of proteasome trajectories (x axis distance, y axis time) generated from 180-s time-lapse images (90 frames; 0.5 frames/s), showing bidirectional movement of proteasomes along neurites (marked by numbers), and the trajectories of individual retrogradely (red) and anterogradely (black) moving as well as stationary (not shown) proteasomes. Summary of frequencies (B) and velocity (C) of MV151-labeled proteasomes at three different stages from all experiments similar to that shown above. Data represent mean ± SEM (n = 20–35 cells; ****p < 0.0001; n.s., not significant; compared with stage 1 neurons, ANOVA with Dunnett’s post hoc test).

(D–F) Proteasome accumulation at the axonal tip after dynein inhibition. (D) Images of 2 DIV neurons immunostained with antibodies specific for proteasome subunit Rpt1, showing local Rpt1 accumulation at neurite tips after bath application of EHNA (0.5 mM; top) or Ciliobrevin D (Cilio D, 0.5 mM; top) for 2 hr, or ectopic expression of p50/dynamitin (p50-GFP; bottom) or dominant-negative C-terminal deleted Lis1 (GFP-ΔC-Lis1; bottom) for 24 hr. Insets are boxed regions of axon growth cone at a higher magnification. ax, Tau-1 positive axon; den, dendrite. Arrows, proteasome accumulation at neurite tips. Bar represents 25 μm. (E) Graph depicts summary of the immunostaining intensities of endogenous proteasome subunits Rpt-1 and PA28α in the most distal 18 μm region (including growth cone) of the axons obtained from the above experiments (average of 20–25 cells; each data points were normalized to the average intensity of the first 5 μm). (F) Summary of quantitative measurements of Rpt1 intensity (±SEM, n = 20–25, compared with control set; **p < 0.001, Dunnett’s test) in neurite tips at two different stages from all experiments similar to that shown above.

(G–I) Representative images and kymographs from ectopic p50-GFP or GFP-ΔC-LIS1 expressing neurons at 3 DIV (G), showing reduced retrograde proteasome transport and unaffected anterograde transport (H). Dashed line marks the longest neurite subjected to kymograph analysis. Histograms in (H) represent average number of directional events (±SEM, 20–35 cells each; **p < 0.001, compared to GFP control, Dunnett’s test) of mobile MV151-labeled proteasomes. (I) Summary of average axon length (±SEM, n = 90; *p < 0.01, **p < 0.001, compared with control, Dunnett’s test) of control and EHNA/Cilio D-treated neurons, as well as the relative neurite length (±SEM, from three independent experiments, n = 30 cells each; ***p < 0.0001, compared with control GFP, Dunnett’s test) of neuron expressing p50-GFP, ΔC-Lis1, or control GFP at 3 DIV.
reached ~1.7- to 3.0-fold of the average amount in the remaining neurites (Figures 2D–2F and S2D). EHNA or Cilio D treatment also led to short Tau-1-positive axon formation (Figure 2I). Genetically, overexpressing p50/dynamin or a C-terminal-truncated lissencephaly protein (ΔC-Lis1, which lacks dynein-binding domain), which disrupts dynein-dynactin function in a dominant-negative way (Sasaki et al., 2000; Tai et al., 2002; Tsai et al., 2005), led to reduced retrograde proteasome transport and enhanced proteasome accumulation at stage 2 nascent axon, as well as defective neurite outgrowth and short axon phenotype (Figures 2G–2I and S2F). Immunostaining the endogenous Par6 revealed that EHNA/Cilio D treatment abolished the accumulation of Par6 at axon tips (Figures S2G and S2H), a physiological abnormality in axon development. Thus, it appears that dynein-dependent proteasome retrograde movements are required for not only differential proteasome abundance, but also axon growth and Par6 accumulation.

**BDNF/cAMP Specifically Promotes Retrograde Proteasome Transport in Axons**

The enhanced retrograde transport of proteasomes observed above emerged spontaneously in a homogeneous culturing environment (Figures 2A–2C) and was found required for axon growth and development (Figures 2D–2I and S2). In vivo, axon development is often guided by environmental cues. To examine whether enhanced retrograde transport can be regulated by external stimuli, we first monitored the dynamics of MV151-labeled proteasomes in response to a bath application of axon-promoting signals, e.g., autocrine factor BDNF or cAMP. Cells were treated with BDNF (50 ng/ml) or the cAMP-elevating drug forskolin (20 μM). In polarized cells, we found that BDNF or forskolin treatment increased the frequency of retrograde transport in the longest process (the nascent axon), without significantly affecting anterograde transport (Figures 3A, S3A, and S3E–S3J). In unpolarized cells, forskolin indeed caused a reduction of proteasome abundance at growing neurite tips, as indicated by the changes of the fluorescence intensity of GFP-fused proteasome subunit Rpt1 (see Figure S3B).

Next, we examined how protein degradation responds to gradients of axon-promoting signals. We coated substrates with stripes of BDNF or cAMP and monitored GFPu degradation in hippocampal neurons cultured on these substrates. We found that cells exhibited a higher rate of polarization (Figure S3C), with both the GFPu intensity and neurite extension rate higher at neurites grown on the stripes than those off the stripes (Figures S3C and S3D). A similar axon-promoting effect was also observed in cells exposed to stripes of proteasome inhibitor lactacystin (Figure 3B). Thus, environmental cues can regulate protein degradation or retrograde proteasome transport to enhance neuron polarization and the growth of nascent axon.

The observed BDNF/cAMP-induced effects were specific to BDNF/TrkB or cAMP/PKA signaling and depended on dynein activity, as they were suppressed by tyrosine kinase inhibitor K252a or specific PKA inhibitor KT5720 (200 nM) (Figure 3A), as well as by a bath-application of 0.5 mM dynein motor inhibitor Ciliobrevin (Figure 3B). Furthermore, no significant changes were observed in either frequency or directionality of the anterograde KIF5B kinesin (tagged with luminol) that cotransported with MV151-labeled proteasome puncta in axons (Figures S3H–S3J), suggesting that axon-promoting signals specifically increase retrograde transport of proteasomes, primarily at the expense of accumulated populations but not kinesin-mediated transport, to facilitate the proteasome removal from axonal terminals.

**BDNF/cAMP Reduces Protein Degradation in Axons**

A consequence of the BDNF/cAMP-induced proteasome removal from the axonal tip is a local increase in protein stability. We applied a compartmented pulse-chase labeling technique to determine the protein half-life ($t_{1/2}$). Newly synthesized proteins were labeled with L-azidohomoaoline (AHA) for 3 hr in both axonal and somatodendritic compartments, followed by a sequential chase at 12, 24, and 36 hr. Consistent with the differential proteasome abundance between axon and dendrites (Figures 1D–1G), half-life of newly synthesized proteins in the somatodendritic region was significantly shorter than that at the axonal tip (Figures S4A and S4B). Such difference was enhanced by axon-promoting signals, as shown in Figure S4B where BDNF treatment (50 ng/ml) prolonged the half-life in the axon tip from 12 to 22 hr, which was suppressed by pretreating cells with TrkB inhibitor K252a, cAMP/PKA inhibitor KT5720, or dynein inhibitor EHNA, suggesting that the prolonged half-life was specifically due to changes in transport.

Likewise, bath application of BDNF or cAMP to stage 3 neurons induced an increase in GFPu intensity at the tip of axon but not dendrites, and this effect did not depend on protein synthesis since it was not suppressed by bath application of protein synthesis inhibitor cycloheximide (Figures S4C and S4D). Together, translation-independent effects of BDNF and cAMP on the protein stability were concomitant with their promoting effects of axonal retrograde transport of proteasomes.

**BDNF/cAMP Facilitates Proteasome Binding to Retrograde Dynein Complexes**

Without significant changes in kinesin-mediated transport, the BDNF/cAMP-induced enhancement of retrograde proteasome transport can occur in two ways: changes in microtubule polarity or changes in dynein-proteasome association. To examine the first possibility, we simultaneously analyzed microtubule polarity and proteasome transport in 2 DIV hippocampal neurons. Cells were infected to induce lentivirus-based expression of GFP fused plus-end-binding protein 3 (EB3-GFP), followed by 1-hr MV151 labeling. Kymographs of EB3-GFP comets in both unpolarized (stage 2) and polarized (stage 3) neurons showed a uniform microtubule polarity, with the majority ($\geq 90\%$) of plus ends oriented distally (see Figures 3C and 3D). Thus, the preferential increase of retrograde proteasome transport was not due to changes in microtubule polarity.

Next, we examined the possibility that dynein-proteasome association was changed by BDNF/cAMP treatment. A potential mechanism is activating adaptors that associate dynein with proteasomes, such as the brain-enriched, proteasome-associated protein “extracellular mutant 29” (Ecm29). Ecm29 interacts with microtubule-associated motor proteins, including Kinesin light chain (KLC) and KIF1B (Gorbea et al., 2004, 2010). To ascertain whether Ecm29 was involved in preferential retrograde proteasome transport, we examined proteasome-dynein complex formation in mouse brain lysates using MV151 labeling and
sucrose-gradient sedimentation. We found that 19S proteasome regulatory cap (Rpt1), dynactin subunit DCTN1, and Ecm29 were co-fractionated in heavier fractions (>19S), while anterograde protein KLC and endosomal protein Rab5 were co-fractionated with a subset of 20S proteasome α and β subunits in lighter fractions (<5S). These data suggest that 26S proteasome and adaptor Ecm29 were associated with retrograde dynein complexes (Figure S5), while the anterograde kinesins primarily interacted with individual proteasome subunits or vesicle-associated subcomplexes (see Figure S5A, as in previous report; Otero et al., 2014). In 3-DIV stage 3 neurons, confocal fluorescence microscopy further revealed that DCTN1 exhibited a higher colocalization with endogenous proteasome subunit Rpt5 in the growing axon than other neurites (Figure S5B), and its physical association with MV151 labeled proteasomes was confirmed by co-immunoprecipitation (Figure S5C).

Western blot also revealed a BDNF-enhanced Ecm29 binding to proteasomes and/or dynein complexes in HEK293 cells co-expressing flag-tag Ecm29 and BDNF receptor TrkB (HEK293 cells cannot respond to BDNF unless co-transfected with the receptor TrkB; Figures 4A and 4B); such enhancement was abolished in cells pretreated with the TrkB or PKA inhibitor, K252a, or KT5720 (Figures 4A and 4B). In vivo, dynein often forms complexes with dynactin to associate with other molecules. We thus assessed whether forskolin treatment modulates the association of Ecm29 with the p50/dynamitin subunit of the dynactin complex. Although the binding maybe indirect, immunoprecipitated endogenous Ecm29 exhibited detectable p50/dynamitin binding, while Ecm29-kinesin association was primarily mediated by the tail cargo-binding domains of KIF5B (KIF5BΔN331; Figure 4C). Furthermore, the BDNF/forskolin-enhanced Ecm29 binding was found specific to dynein complexes, as BDNF or...
Figure 4. BDNF/cAMP Facilitates Ecm29 Association with Proteasomes and Dynein Complexes

(A) BDNF increased Ecm29 binding with endogenous proteasomes and dynein complexes. 293T cells were co-transfected with TrkB and FLAG-tagged Ecm29. After 18-hr transfection, cells were bath applied with and without BDNF (50 ng/ml) for 1 hr (K252a or K252b was applied 30 min prior to BDNF), and cell lysate was immunoprecipitated by FLAG M2 antibody and blotted with antibodies targeted to Ecm29 (“FLAG-Ecm29”), phospho-(Ser/Thr) PKA substrates (“p-FLAG-Ecm29”), dynactin subunit 1 (“DCTN1”), dynein intermediate chain 1 (“DIC1”), and proteasome 19S Rpt1 subunit (“Rpt1”), together with MV151-labeled proteasome subunits (“b1, b2, and b5”). (Histograms) Summary of quantitative measurements of BDNF-induced changes in the level of MV151 signals and DIC1, normalized to the band intensity measured from the corresponding immunoprecipitated FLAG-Ecm29 (±SEM, n = 3; **p < 0.001, compared with control, t test).

(B) Similar to (A), except cell lysates were immunoprecipitated by antibodies against KLC1 or DCTN1. (Histograms) Summary of quantitative measurements of BDNF-induced changes in the level of MV151 signals, normalized to the band intensity measured from the corresponding KLC1 or DCTN1 (±SEM, n = 3, compared with untreated control; **p < 0.001, t test).

(C) Similar to (A), except cells were co-transfected with p50-GFP and HA-KIF5B-DN331. Cell lysates were immunoprecipitated by antibodies against Ecm29. Summary histograms below show that bath application of 20 μM forskolin for 15 min significantly increased the level (±SEM, n = 3, compared to untreated control; ***p < 0.0001, t test of Ecm29 binding with p50/dynamitin, but not that with headless KIF5B (HA-KIF5B-DN331).

(D–H) Enhanced interaction of proteasomes with dynein complexes in response to BDNF/TrkB and cAMP/PKA signaling in developing neurons. (D) Confocal images of cultured hippocampal neurons co-immunostained on 3 DIV for DIC1 and proteasome subunit Rpt3, showing an increase in the co-localization (yellow, arrows) of Rpt3 staining (green) and DIC1 staining (red) after 1 hr bath application of BDNF. (E) Similar co-immunostaining and treatments procedure as in (B), except 3D SIM images were acquired and analyzed for 3 DIV neurons. This is a representative surface rendering 3D images sampled from the distal end (20 μm) of axons showing motor-protein-positive puncta (DIC1 or KIF5B, green) and the calculated motor-proteasome co-localized surfaces (yellow, indicated by arrow). The volume thickness is 3 μm. (F) Average percentage volume (±SEM, n = 8–10 cells for each set of experiments; t test) of proteasome subunits co-localized with motors in the first (“proximal”) or last (“distal”) 20 μm region of axons. (G and H) Summary graphs showing the percentage volume (±SEM, n = 5–10 cells for each set of experiment; **p < 0.001, ***p < 0.0001; t test) of Rpt3/Rpt5 and KIF5B/Rpt5 co-localized with DIC1/KIF5B in 3 DIV cultures that were exposed to BDNF (50 ng/ml, 30 min) or cAMP-elevating agent forskolin (20 μM), with and without pretreatment with K252a (200 nM), K252b (200 nM), or KT5720 (200 nM).
forkolins treatment did not significantly affect or compete the formation of anterograde kinesin-proteasome-Ecm29 complexes (Figures 4B and 4C), consistent with the observation that axon-promoting effects of BDNF/cAMP signaling primarily acted on retrograde proteasome transport (Figures 3A, 3B, S3A, and S3H).

We used confocal imaging and super-resolution 3D structured illumination microscopy (3D SIM) to confirm BDNF/cAMP-induced enhancement of proteasome-dynein complex formation on axon in 3-DIV stage 3 neurons. As shown in Figure 4D, co-immunostaining of endogenous proteasome subunit Rpt3 and cytoplasmic dynein intermediate chain subunit (DIC1) in untreated control cells revealed a punctate pattern along the neurite with 16% of Rpt3 co-localizing with DIC1, and this Rpt3-DIC1 co-localization was increased to 30% after bath application of BDNF or forskolin. The increased Rpt3-DIC1 complexes in the presence of BDNF were validated by co-localization analysis obtained from 3D SIM images. The number and the volume of Rpt3 co-distributed with DIC1 were significantly increased in the presence of BDNF or forskolin (Figure 4D).

Such promoting effects were completely abolished by pretreating cells with TrkB or PKA inhibitor (K252a or KT5720; Figure S7B). In contrast, the level of KIF5B kinesin co-distributed with proteasome subunit Rpt5 was unaffected by BDNF treatment (Figures 4E and 4H), confirming that BDNF signaling selectively enhances recruitment of proteasome to retrograde motor, without affecting the anterograde complexes (Figures 3A, 3B, S3A, and S3H).

Adaptor Protein Ecm29 Is Required for Proteasome Transport and Axon Growth

The BDNF/cAMP-enhanced retrograde proteasome transport can be mediated by multiple pathways. To ascertain whether dynein-Ecm29-proteasome interaction is solely responsible for such an enhancement, we expressed ectopically N- and C-terminal truncated forms of Ecm29 (denoted as Ecm291320–1840, Ecm291032–1840, and Ecm291–1039), which are defective in proteasome and dynein association, respectively (see Figure S5D) (Gorbea et al., 2010). We found that ectopic expression of either N- or C-terminal-truncated Ecm29 significantly reduced the level of mobile MV151-labeled proteasomes, as compared with cells expressing WT Ecm29 (Figure 5A), indicating that both proteasome and motor interaction domains of Ecm29 are required for proteasome transport.

Downregulation of Ecm29 by specific shRNA (Figure S6) for 3 days yielded similar effects in reducing proteasome transport (Figure 5A). Moreover, neurons expressing truncated Ecm29 or Ecm29 shRNA displayed short axons and polarization defects, while Ecm291320–1840 and Ecm291–1039 expressing neurons exhibited a significant increase of no-axon (NA) cells, similar to neurons expressing dynein-disrupting p50/dynamitin or treated with dynein inhibitor (Figures 2F, 5B, and S2F). Thus, both proteasome transport and proper axon development depend on intact Ecm29 in vitro and in vivo.

BDNF/cAMP Promotes Retrograde Proteasome Transport via Ecm29 Phosphorylation

If BDNF/cAMP-enhanced retrograde transport is due to activation of the adaptor Ecm29, one expects to see modification of this protein in response to BDNF/cAMP treatment. In eukaryotes, most protein activations occur through phosphorylation. To ascertain whether BDNF and cAMP signalings phosphorylate Ecm29, we screened for potential phosphorylation sites of Ecm29 by liquid chromatography-tandem mass spectrometry and identified two BDNF/cAMP-responsive phosphorylation sites, Ser1414 and Ser1661, at the C terminus of Ecm29 (Figure S7A). The specificity of BDNF/cAMP regulation of Ecm29 phosphorylation was confirmed by immunoprecipitation of flag-tagged Ecm29 in cells treated with BDNF or forskolin; such phosphorylation disappeared if cells were pretreated with TrkB or PKA inhibitor (K252a or KT5720; Figure S7B). In addition, immunofluorescence staining with specific antibodies against Ecm29 or its phosphorylated form Ecm29S1414 or Ecm29S1661 showed that these proteins were primarily accumulated at axons but not dendrites (Figures 6A, 6B, and S7C).

To ascertain whether Ecm29S1414/S1661 phosphorylation is responsible for the enhanced proteasome-dynein association, we assessed proteasome-Ecm29-motor association by western blotting of immunoprecipitated FLAG-Ecm29 from HEK293 cells co-expressing BDNF receptor TrkB with WT Ecm29 (Ecm29WT) or Ecm29 phosphorylation variants, i.e., Ecm29S1414A/S1661A and Ecm29S1414D/S1661D. As expected, cells expressing phosphorylation-mimicking Ecm29S1414D/S1661D exhibited a higher binding affinity to both proteasome and dynein than Ecm29WT (Figures 6C and 6D). In contrast, cells expressing phosphorylation-deficient Ecm29S1414A/S1661A were unresponsive to BDNF/cAMP-enhanced interaction of either proteasome or dynein, indicating that Ser1661 and Ser1414 phosphorylation is solely responsible for promoting Ecm29-mediated proteasome-dynein interaction (Figure 6C). Likewise, an ectopic expression of Ecm29S1414A/S1661A reduced the percentage of retrograde movements (Figures 6E and 6F). Thus, BDNF/cAMP signaling facilitates the formation of proteasome-dynein complexes through Ecm29 phosphorylation, leading to increased retrograde transport of proteasomes in growing axons.

Stage-Dependent Ecm29-Mediated Proteasome Transport Can Be Explained by a Simple Model

The results above suggest that axon-promoting factors phosphorylate Ecm29 to enhance local protein stability (Figure S4), as a local and global positive regulation manifested in the differential proteasome abundances between axon and dendrite tips. Having sustainable axon growth requires cells to maintain such asymmetry over long axons in a varying environment, which has been a caveat in conventional Turing reaction-diffusive scheme (Turing, 1952), as Turing model predicts cells to form multiple positive regulation sites when cell size is beyond a certain length scale. In this study, however, we observed that the frequency of retrograde proteasome transport increased with the axon lengths (Figures 2A and 2B). Theoretically, the longer the axon, the more non-specific signals the cells could experience from the environments. To gain a theoretical understanding on how the referential transport depends on axon length and the strength of specific over nonspecific axon-promoting signals, we developed a simple mathematical model. In this model, we assumed that proteasomes are transported by motor-associated Ecm29 and that Ecm29 switches the associated motor in a phosphorylation-dependent manner (see Supplemental Information for details). While the specific signal was
created by Ecm29 phosphorylation at the axon tip, the non-specific signals were implemented by allowing cells to express non-convertible, constitutive active or defective Ecm29 (i.e., phosphorylation-mimicking or deficient), along with the WT. Using these simple assumptions, the model does produce the spatial profiles of phosphorylated Ecm29, total Ecm29, and proteasomes resembling those found in experiments (Figures 1D, 1E, 7A, and S7C).

Conversely, the model predicted that at the initial stage where the nascent axon has a length similar to other neurites, the
enhanced retrograde proteasome transport created at the axon tip is vulnerable to non-specific signals: overexpressing constitutively active Ecm29 led to MA formation, while cells overexpressing defective Ecm29 failed to form axon (Figure 7B). To test this prediction and to see whether it is important for neuronal development, we used cultures of rat embryonic cortical neurons. Constructs encoding Ecm29WT or one of its mutated forms were in utero electroporated (IUE; Saito and Nakatsuji, 2001) into the developing brain of rat embryos on E17. Cultures were then prepared right after electroporation. Such IUE method was used for transfection of full-length Ecm29 constructs rather than lentiviral-based expression due to the constraint of construct size of the lentivirion. At 3 DIV, the percentage of single axon (SA) cells among Ecm29WT-expressing cortical neurons was comparable to that in non-transfected (control) neurons (Figures 7D). Consistent with the model prediction, however, expressing either Ecm29S1414A/S1661A or Ecm29S1414D/S1661D significantly reduced the SA population. Notably, for the remaining populations, Ecm29S1414A/S1661A expression greatly increased the NA population and reduced neurite number and length, while the Ecm29S1661D/S1414D expression increased the MA population and neurite length, similar to those neurons treated with proteasome inhibitors in previous studies (Cheng et al., 2011a; Yan et al., 2006) (Figures 7D–7F and S1A). We also found polarization defective phenotypes in neurons expressing Ecm29 phosphorylation variants at P3 rat cortices in vivo; such phenotypes can be reduced by co-expressing WT Ecm29WT (Figures S6E–S6G). These results are consistent with the model prediction and
indicate that the initiation of axon strongly depends on specific Ecm29 phosphorylation at Ser1414 and Ser1661.

To further investigate how neuron development depends on specific over non-specific signals, we plated IUE neurons (transfected with Ecm29WT or one of its mutated forms) on substrates stripe coated with BDNF. For neurons with their somata located at the stripe boundary on 3 DIV, we analyzed their distribution of axon initiation site along their soma (Figure 7G). Similar to those expressing Ecm29 mutants in regular cultures (Figure 7C), neurons expressing Ecm29 mutants exhibited altered polarization phenotypes on stripes (Figure 7G). Furthermore, both control and Ecm29WT-expressing neurons showed higher probability of axon differentiation for neurites initiated on the stripe than off the stripe, whereas the axon-promoting effect of BDNF stripes was diminished in neurons expressing Ecm29 mutants (Figures 7G and 7H). Thus, Ecm29 phosphorylation is critical for both spontaneous and BDNF-induced axon formation in cortical neurons. Finally, the model predicts that the cellular capability to maintain the differential effect over non-specific signals increased with the axon length: once the cell passed the initiation stage, its ability to maintain preferential transport increased with the axon length (Figure 7I), which is consistent with the experimental observation that 3-DIV neurons exhibited a higher degree of preferential transport effects (Figure 2).

**DISCUSSION**

During neuronal maturation, redistribution of cytoplasmic constituents and controlled protein turnover are essential for the specification and development of axonal and dendritic compartments. To achieve increasing demand of cytoplasmic constituents for the rapidly extending axon, previous studies have shown that neurons may utilize several distinct mechanisms: (1) selective bulk flow of the cytoplasm into the growing axon (Bradke and Dotti, 1997); (2) directional trafficking of proteins, mRNAs, and organelles to the axon versus dendrites (Horton and Ehlers, 2003; Jung et al., 2014; Kapitein and Hoogenraad, 2011; Namba et al., 2011; Rolls, 2011); (3) preferential stabilization or degradation of growth-associating proteins by selective regulating UPS activity (Cheng et al., 2011a; Yan et al., 2006). In the present study, we demonstrated that the abundance of multisubunit mega-complex proteasomes may be spatially regulated in a manner that contributes to the axon development during early stages of neuronal differentiation. By direct observation of bidirectional trafficking of proteasomes in developing neurons via covalent labeling of active proteasomes with a specific reporter MV151, we found a preferential increase of retrograde proteasome transport that reduced proteasome abundance in the distal end of growing axons, facilitating rapid axon extension. Furthermore, we showed that the enhanced retrograde transport was mediated by phosphorylation of adaptor protein Ecm29 that promotes binding of proteasomes to the dynein motor. Such global regulation of proteasome distribution may represent an efficient mechanism of polarized distribution of proteins in the cytoplasm (Figure 7).

Proteasomes were previously thought to be diffusely distributed in nuclear and cytoplasmic compartments, and the distribution could be dynamically regulated by cytoskeleton-based active transport or adaptor/scaffold protein interactions, during cell fate decision (Chang et al., 2011), growth cone steering, and activity-induced synaptic modification (Bingol and Schuman, 2006; Bingol et al., 2010; Shen et al., 2007). For example, unequal partitioning of proteasomes into two sibling cells of proliferating T lymphocytes generates disparity in the abundance of lineage-determining factors (Chang et al., 2011). We found here that post-mitotic neurons generated patterns of proteasome abundance by biasing retrograde versus anterograde proteasome transport during the early period of active axon growth. This preferential proteasome transport required microtubule-associated dynein motors and could be upregulated by stimulation with axon-promoting BDNF/cAMP/PKA signaling pathway (Cheng et al., 2011b; Shelly et al., 2007), leading to differential protein turnover between the axon and dendrites that are required for local differentiation, metabolic demands, axon pathfinding, and morphological changes of the growing axon. For example, during the early phase of axon initiation, the emerging asymmetry of proteasome transport could generate an intracellular gradient of protein degradation along the nascent axon that facilitates axon specification by accumulating axon determinants at the distal end of the neurite. During axon pathfinding, bath application of proteasome inhibitor abolished axon steering by extracellular guidance factors netrin and semaphorin 3A (Campbell and Holt, 2001). Whether the asymmetry of proteasome activity or distribution exists within the local region of the growth cone during axon steering remains to be further investigated. Asymmetry of proteasome-dependent protein turnover must work in coordination with preferential mRNA transport and local translation (Jung et al., 2014) in order to achieve a myriad of structural and functional changes required for neuronal development.

In addition to the induction of proteasome redistribution, which alters regional capacity of protein turnover non-specifically, BDNF/cAMP/PKA signaling also causes phosphorylation of an E3 ligase Smurf1 that prevented and promoted the degradation of Par6 and RhoA, two proteins having opposite functions in regulating axon growth, respectively (Cheng et al., 2011a). Thus, extracellular regulatory factors may exert both selective and non-selective controls on local proteasome-dependent protein turnover, two complementary mechanisms for changes in protein constituents in axonal versus somatodendritic compartments of developing neurons. Alteration of the proteasome substrate selectivity for positive and negative axon growth modulators could regulate the local levels of these modulators, whereas the redistribution of proteasomes exerts a long-range effect on the global capacity of protein turnover in different neuronal compartments.

In this study, perhaps the most striking observation is that the degree of the preferential transport effect increases with the axon length, by which cell polarity can be sustainable over a long distance in varying environments (Cheng et al., 2011a; Namba et al., 2011). Both our experiments and modeling suggest that this behavior results from transport-mediated removal: the longer the axon, the less the proteasomes at axon tip. Consequently, this phenomenon leads to global asymmetric proteasome redistribution that may be of unique importance for neurons because of the extensively compartmentalized neuronal morphology. It is likely that proteasomes activity and transport plays a critical role in neuronal development as...
Figure 7. Proper Axon Initiation Requires Specific Ecm29 Phosphorylation at Ser1414 and Ser1661

(A) Numerical results show the steady-state spatial profiles of phosphorylated Ecm29 (black), total Ecm29 (blue), and proteasomes (red) along the axon (solid line) or dendrite (dashed line) measured from the soma (coordinate = 0) to the tip.

(B) At the axon initiation stage, numerical results show how the difference of proteasome concentrations at axon and dendrite tips (normalized by the soma concentration) depends on the presence of phosphorylation-mimicking or deficient Ecm29 (normalized to the total amount of Ecm29). When phosphorylation-mimicking (deficient) Ecm29 is overexpressed, cells were predicted to form multiple (no) axons.

See Supplemental Information for the details and the parameters.

(C–F) Ectopic expression of Ecm29 S1414A/S1661A and Ecm29 S1414D/S1661D altered axon formation. (C) Images of cultured cortical neurons expressing various forms of Ecm29. Arrows show axons. Bar represents 25 μm. (D) Bar graphs showing the percentage of neurons (n = 3 cultures, 90 cells each) exhibiting three different polarization phenotypes: SA, NA, or multiple axons (“MA”). Datasets (connected by dashed lines) showing significant difference are marked (*p < 0.05, compared with corresponding sets in WT Ecm29, Dunnett’s test). (E) Summary of the average axon length (±SEM) for the cultures described above in (B) (n = 51–90 cells; *p < 0.05, **p < 0.001, compared with WT Ecm29, Dunnett’s test). (F) Composite tracing of axons from 25 randomly sampled 3 DIV neurons expressing various forms of Ecm29.

(G and H) Ecm29 phosphorylation on Ser1414 and Ser1661 affected BDNF-induced axon initiation. (G) Images of cortical neurons expressing various forms of Ecm29 with the axon initiated on or off the BDNF-coated stripes. Arrows show axons. Stripe width represents 50 μm. (H) Bar graphs of results from all experiments, showing the percentage of axons that were initiated on or off the stripes coated with BDNF. Only neurons with the soma located at the stripe boundary were counted. Data represent as mean ± SEM (n = 3, 30 cells each, **p < 0.001, t-test).

(legend continued on next page)
well as during regeneration and disease states, where there are asymmetric demands for protein turnover at distant parts of the neuron. The modulation of proteasome interaction with adaptor proteins for transport motors shown here represents one of many potential mechanisms for regulating proteasome redistribution within the neuronal cytoplasm. For example, the development of the cytoplasmic transport barrier at the axon initial segment in mature neurons (Song et al., 2009) as well as changes in the motor protein efficacy and microtubule-associated proteins could all play important roles in transport-dependent regulation of local proteasome abundance in neurons.

EXPERIMENTAL PROCEDURES

Animal protocols were approved by the Academia Sinica Institutional Animal Care and Utilization Committee.

Cell Culture Preparations and Immunostaining

Hippocampal neurons were prepared from rat embryos on E17 as previously described (Dotti et al., 1988) and were cultured in neurobasal medium supplemented with Gem21 NeuroPlex (GEMINI bio-products). A similar procedure was applied to the preparation for cortical neuronal cultures. Human Embryonic Kidney 293T cells used for biochemical assays were cultured in Dulbecco’s modification of Eagle’s medium supplemented with 5% fetal bovine serum (BSA) (Sigma). Transfection of these cultures was performed using lentivirus-based expression system or Lipofectamine 2000 (Invitrogen) according to the manufacturer’s Instructions. Unless otherwise stated, hippocampal neurons were used as a standard model for in vitro immunocytochemistry to analyze axon/dendrite differentiation. Cortical neuronal cultures were used for obtaining enough cells for biochemical assays that do not need transfection of exogenous proteins.

For immunostaining, cultured hippocampal neurons were fixed with 4% paraformaldehyde for 12 min and then permeabilized in 0.3% Triton X-100 for 20 min and blocked with 1% BSA for 1 hr. The fixed cells were processed further for immunostaining according to standard procedure and imaged with a confocal microscope (Zeiss LSM700) equipped with a 63x oil-immersion objective (NA1.0). Images were analyzed and processed for presentation in the figures, using brightness and contrast adjustments with NIH ImageJ software and following the guideline of Rossner and Yamada (2004). Colocalization of Rpt3-DIC1 or Rpt5-KIF3B was evaluated using structured illumination microscopy (Zeiss ELYRA PS1) equipped with Plan Apo 63x oil-immersion objective (NA1.4), and raw images were acquired with a total magnification of 79 nm per camera pixel with 2 spacing of 125 nm between planes. Images were analyzed and processed using ZEN software (Zeiss) and Imaris software (Bitplane). Z correction was applied to entire 3D image datasets.

Plasmids, Antibodies, and Materials

Detailed information is described in the Supplemental Experimental Procedures.

Live-Cell Imaging and In-Gel Detection of Labeled Proteasome Subunits

The fluorescent labeling of proteasomes were performed as described previously (Verdoes et al., 2006a). For live-cell imaging experiments, neurons were bath incubated with 100 μM MV151/MV152 for 1 hr at 37° C in culture medium and visualized with a 63x oil-immersion objective (NA1.4; Zeiss) on a Zeiss Observer.Z1 integrated with a camera AxioCam MRm and a temperature/CO2 module. Zen blue (Zeiss) software was used for microscope control and data acquisition. MV151 and MV152 were excited at λex = 543 nm and were detected at 560–620 nm. For in-gel MV151-labeled proteasome detection, cell lysate prepared in native gel lysis buffer (20 mM Tris-HCl [pH 7.4], 5 mM MgCl2, 1 mM DTT, and 2 mM ATP) was incubated for 1 hr at 37° C with MV151 (100 μM). Reaction mixtures were boiled with Laemmli’s buffer containing β-mercapto-ethanol for 12 min and were resolved on 12% SDS-PAGE. In-gel visualization was performed in the wet gel slabs directly on the Typhoon FLA9000 laser scanner (λex = 532 nm, λem = 560; GE Healthcare Life Sciences).

Statistics and Modeling

To decide the statistical test for the comparison between two datasets, we first examined whether the data in each set are normally distributed, using Jarque-Bera test. For datasets with normal distribution, t test was used. For comparison involving multiple datasets, one-way ANOVA test was used followed by post hoc Tukey test. Details for the numerical model and parameters for simulations can be found in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.develcel.2015.10.018.

AUTHOR CONTRIBUTIONS

M.-T.H. provided hippocampal cultures and participated in gene expression, live-cell imaging, and 3D SIM experiments. A.Y.L. conceptualized, performed, and analyzed the biochemistry experiments, plasmid constructions, and in vitro electroporation and co-wrote the manuscript. T.-Y.C. performed and analyzed the in utero electroporation experiments. Y.-L.W. performed and analyzed 3D SIM imaging. M.-C.N. performed and analyzed sucrose gradient experiments. B.I.F. and H.S.O. provided synthetic proteasome reporters. C.-L.G. developed the model and co-wrote the manuscript. P.-L.C. supervised, conceptualized, performed, and analyzed biochemistry, live-cell imaging, and in vitro electroporation experiments, and co-wrote the manuscript. All authors read and contributed to the manuscript.

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