Regulation of Intrinsic Axon Growth Ability at Retinal Ganglion Cell Growth Cones

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PURPOSE. Mammalian central nervous system neurons fail to regenerate after injury or disease, in part due to a progressive loss in intrinsic axon growth ability after birth. Whether lost axon growth ability is due to limited growth resources or to changes in the axonal growth cone is unknown.

METHODS. Static and time-lapse images of purified retinal ganglion cells (RGCs) were analyzed for axon growth rate and growth cone morphology and dynamics without treatment and after manipulating Kruppel-like transcription factor (KLF) expression or applying mechanical tension.

RESULTS. Retinal ganglion cells undergo a developmental switch in growth cone dynamics that mirrors the decline in postnatal axon growth rates, with increased filopodial adhesion and decreased lamellar protrusion area in postnatal axonal growth cones. Moreover, expressing growth-suppressive KLF4 or growth-enhancing KLF6 transcription factors elicits similar changes in postnatal growth cones that correlate with axon growth rates. Postnatal RGC axon growth rate is not limited by an inability to achieve axon growth rates similar to embryonic RGCs; indeed, postnatal axons support elongation rates up to 100-fold faster than postnatal axonal growth rates. Rather, the intrinsic capacity for rapid axon growth is due to both growth cone pausing and retraction, as well as to a slightly decreased ability to achieve rapid instantaneous rates of forward progression. Finally, we observed that RGC axon and dendrite growth are regulated independently in vitro.

CONCLUSIONS. Together, these data support the hypothesis that intrinsic axon growth rate is regulated by an axon-specific growth program that differentially regulates growth cone motility.

Keywords: axon, regeneration, CNS, growth cone, filopodia, lamellipodia, KLF

In the mammalian central nervous system (CNS), injured or diseased axons fail to regenerate successfully and the neuron often dies or atrophies.¹ This failure is due to both intrinsic² and extrinsic factors.^{3–5} Most efforts to improve CNS axon survival and regeneration have focused on extrinsic factors, such as overcoming glial-associated inhibitors^{6–9} or increasing deficient neurotrophic factor signaling.^{10–15} Recent studies show axon regeneration can be promoted by differentially regulating intrinsic signaling molecules like PTEN,^{16,17} SOCS3,¹⁸ and the Krüppel-like family (KLF) family of transcription factors,^{19,20} among others.²¹ However, it remains unknown how these molecular manipulations regulate the mechanisms regulating axon growth, such as the motile dynamics of the axonal growth cone.

Growth cones, the motile tips of axons, direct the temporal and spatial patterning of the nervous system by regulating the rate and the direction axons grow.²² To regulate axon growth,

growth cones differentially alter the protrusion of two actin-based structures, lamellipodia and filopodia,²³ in response to signaling from cellular²⁴ and extracellular matrix guidance cues,²⁵ integrated with intrinsic growth states.²⁶ Identifying the specific, physiologically relevant changes in lamellar and filopodial protrusive activity that regulate the direction and the rate of growth cone advance in response to cues has revealed specific mechanisms that locally regulate lamellar and filopodial protrusion,²⁵ growth cone guidance,²⁷ and neurite growth rate.²⁸ Hence, the molecular regulation of axon growth is assumed to be at the growth cone.²⁹ However, whether intrinsic changes during neuronal development or molecular manipulations in the adult that promote axon regeneration impinge on growth cone-specific mechanisms has not been addressed.

Here we show that the decline in postnatal retinal ganglion cells' (RGCs') ability to grow axons rapidly is associated with a coherent program of developmentally and KLF-regulated

changes in axonal growth cone morphology and protrusive dynamics. A concomitant increase in both growth cone pausing and retraction, as well as a decreased ability to achieve rapid instantaneous rates of forward progression largely explain slowed axon growth rate in postnatal RGCs, despite sufficient cellular resources to support growth at rates up to 100-fold faster under applied tension. These changes at the growth cone are mirrored by the observation that developmental growth programs regulate axon and dendrite growth independently. Together, these data support the hypothesis that axon-specific growth programs regulate intrinsic axon growth ability at the growth cone.

MATERIALS AND METHODS

Animals

Experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Miami Institutional Biosafety Committee and the Institutional Animal Care and Use Committee.

Retinal Ganglion Cell Purification and Culture

Retinal ganglion cells were purified from male and female embryonic day 13 (E13) to postnatal day 8 (P8) Sprague-Dawley rats (Harlan Laboratories, Tampa, FL, USA) by immunopanning³⁰ and cultured on poly-D-lysine (70 kDa, 10 µg/mL; Sigma-Aldrich Corp., St. Louis, MO, USA) and laminin-coated (2 µg/mL, Sigma-Aldrich Corp.) MatTek dishes (P35G-1.0-2-C; MatTek Corp., Ashland, MA, USA), as described.³¹ In some experiments, P5 RGCs were used instead of P8, as the P5 retinas are slightly easier to dissociate, resulting in a larger yields per prep. However, both P5 and P8 RGCs elongate axons at similar rates in culture.²

Mechanical Tension

Force was applied to RGC growth cones with calibrated glass needles coated with concanavalin A (10 mg/mL in PBS), as previously described for peripheral³² and CNS neurons.³³ Briefly, two needles were mounted in a micromanipulator; one needle was calibrated for its bending constant and used as a pulling needle applied to the axon, and the other needle was used as an unloaded reference for bending of the towing needle. A calibrated needle, with a bending modulus between 5.1 and 15.4 p dyn/rm, was attached to an RGC's growth cone and the axon was pulled in three 30-minute steps by applying a constant force. The starting tension was between 50 and 100 p dyn and maintained by moving the micromanipulator to maintain the proper deflection of the calibrated needle. Subsequent steps were between 50 and 100 p dyn higher than the previous tension. Pulled growth cones were recorded as described below and analyzed for neurite length and tension as described.³⁴

Tissue Processing and Visualization

Cultured RGCs were fixed with 4% paraformaldehyde in PBS, washed with PBS, and then extracted with 0.5% Triton X-100 for 10 minutes in antibody buffer,³¹ conditions that preserve microtubule filaments but washout tubulin monomers.³² Extracted and washed cells were immunostained with the nuclear marker 4',6-diamidino-2-phenylindole (1:3000; Invitrogen, Grand Island, NY, USA), the axonal marker β III-tubulin (tuj1, 1:350, MRB-435P; Covance, Princeton, NJ, USA) and the dendrite marker MAP2 (1:10,000, ab5392; Abcam, Cambridge, UK), and visualized with Alexa 488 or 594 secondary

antibodies (1:1000; Invitrogen). All images were processed identically in Photoshop (Adobe Systems, Inc., San Jose, CA, USA) from experiments conducted in parallel to enhance contrast for easier visualization during publication without altering data integrity.

Axon and Dendrite Growth Analysis

Acutely purified embryonic or postnatal RGCs were cultured for 3 days in full Sato medium as above, fixed, and immunostained for β III-tubulin and MAP2 as above. Neurite length was quantified using the ImageJ Plugin, Neurite Tracer (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Axon length was defined as the total neurite length minus the total dendrite length. ANOVA and linear regression statistical analyses were performed with SPSS Statistics 19 (IBM, Armonk, NY, USA).

Time-Lapse Microscopy and Analysis

During time-lapse recordings, full-Sato volume was increased to 2.7 mL. Cultures were maintained at 37°C, 10% CO₂, with a heated and humidified stage (Zeiss, Thornwood, NY, USA) and recorded with a Zeiss camera at 12 frames per minute with differential interference contrast (DIC) optics (Plan Apo 63x/1.40 DM objective; Zeiss). To quantify axon growth rate, growth cone rate traces for both E20 and P8 growth cones were generated by locating the center of the growth cone in each video frame and then calculating the displacement at each time point. To calculate the contribution of instantaneous growth cone advance to the overall axonal growth rate, the growth rate of advancing growth cones was averaged, discarding periods of growth cone pause or collapse. The contribution of growth cone pause and collapse was then calculated by subtracting the contribution of instantaneous growth cone advance from the total growth rate. To quantify growth cone motility, we selected the first three growth cones from each experimental group, E20, P5, P5^{KLF4}, and P5^{KLF6}, that met the following criteria: (1) the recorded period lasted at least 60 minutes, (2) the recording was sufficiently clear to visualize all motile elements over the recorded period, and (3) the recorded neuron did not contact another cell. To compare data from growth cones differing in both size and activity levels, data from individual growth cones were normalized by expressing the values as a percentage of embryonic RGCs. Growth cone protrusions were analyzed as previously described.²⁷ To analyze axon growth rate in these experiments, the first three E20 or P5 growth cones encountered were recorded for at least 1 hour.

Lentiviral Transfection

Retinal ganglion cells were transduced as previously described.¹⁹ Lentiviral stocks, amplified at the University of Miami viral vector core and carrying either KLF4 or KLF6 (Open Biosystems, GE, Piscataway, NJ, USA), were diluted to 1.5 to 4.5×10^7 IU per mL, directly into P5 RGC cultures 6 hours after immunopanning and plating on MatTek dishes as above. The cells were incubated at 37°C, 10% CO₂ for 5.5 hours. The virus-containing supernatant was removed by aspiration, the cells were washed twice with full Sato, and then incubated for 3 days with media changes every 24 hours before imaging and analyzing as above. Using these viral transductions, approximately 80% of the RGCs are transduced with detectable changes in KLF gene expression (not shown).

FIGURE 1. Growth cone lamellar and filopodial dynamics are intrinsically regulated in embryonic and postnatal RGCs. Time-lapse sequences show that (A) E20 RGC growth cones are phenotypically lamellar protruding numerous large, sheet-like lamellipodia (L, arrow) between filopodia (f), whereas (B) P5 RGC growth cones are phenotypically filopodial, protruding fewer smaller lamellipodia between filopodia. Time in seconds is indicated. Scale bar: 5 μm. Normalized to E20, (C) P5 axons grew significantly slower than E20 axons. (D) P5 growth cones initiated lamellae less frequently that were smaller in area and traveled shorter distances, although lamellar protrusion rates were similar. (E) In P5 growth cones, filopodia initiated less frequently and protruded at slower rates, the time bound to the substrate was longer, rigidity while bound to the substrate was reduced, and lifetime was increased. Non-normalized means are indicated above each bar. * $P < 0.001$, ** $P < 0.05$, $n = 3$ per age.

RESULTS

Axonal Growth Cone Protrusion Is Intrinsically Regulated in RGCs

The intrinsic capacity of RGCs for rapid axon growth declines progressively shortly after birth.² To determine if axon growth rate is related to changes in axonal growth cone structure or motility, growth cone protrusive activity and short-term (1- to 4-hour) axonal growth rates were analyzed in time-lapse recordings of E20 and P5 RGC growth cones growing on a laminin substrate (Fig. 1). Qualitatively, E20 growth cones were phenotypically lamellar, extending numerous, large sheet-like lamellae between filopodia (Fig. 1A). In contrast, postnatal growth cones were smaller and phenotypically filopodial, extending few, small lamellipodia (Fig. 1B). Under time-lapse conditions, E20 RGCs elongated at rates approximately nine times faster than P5 axons (Fig. 1C), consistent with our previous data.² Thus, qualitatively, embryonic and postnatal growth cones are phenotypically distinct with markedly reduced lamellar protrusion in the growth cones of slow-growing postnatal axons.

Lamellar and Filopodial Dynamics Are Intrinsically Regulated

Individual lamellae protruding between filopodia in E20 and P5 RGCs were quantitatively analyzed for lamellar initiation frequency, mean lamellar area, protrusion distance, and protrusion rate (Fig. 1D). In E20 growth cones, lamellae initiated 4-fold more frequently. These E20 lamellae were significantly greater in area and protruded longer distances compared with P5 growth cones, despite similar protrusion rates at both ages. Thus, in cultured RGCs, postnatal RGCs

protrude smaller lamellae, and less frequently compared with embryonic RGCs.

Individual filopodial protrusions were quantitatively analyzed in E20 and in P5 RGCs for initiation frequency, protrusion rate, the time filopodia were bound to the substrate, the time filopodia retained a rigid phenotype while bound to the substrate (filopodial rigidity), and the overall lifetime (Fig. 1E). In E20 growth cones, filopodia initiated more frequently than in P5 growth cones. E20 filopodia protruded over two times faster than P5 filopodia and remained bound to the substrate over three times shorter than P5 filopodia. Filopodial rigidity, the percentage of the time bound that filopodia maintain a straight, unbent or unkninked, morphology,²⁷ also differed at both ages, with E20 filopodia maintaining rigidity 99% of the time, whereas P5 filopodia were rigid only 56% of the time. Finally, consistent with decreased time bound to the substrate, E20 filopodial lifetimes were significantly shorter than P5 filopodial lifetimes. Thus, filopodial dynamics also are intrinsically regulated. Postnatal growth cones protrude long-lived filopodia with increased substrate adhesion that fail to retain their rigidity, consistent with previous studies showing that increased adhesion in structurally compromised filopodia fails to support lamellar protrusions.²⁷

To determine if enhancing or suppressing RGCs' intrinsic capacity for axon growth alters lamellar or filopodial dynamics, we expressed either the axon growth suppressor KLF4 (P5^{KLF4}) or the axon growth enhancer KLF6 (P5^{KLF6}) in P5 RGCs. Qualitatively, P5^{KLF4} growth cones were phenotypically filopodial and similar to P5 growth cones, protruding few, small lamellae (Fig. 2A). P5^{KLF6} growth cones were also filopodial, but P5^{KLF6} growth cones also protruded large lamellae that appeared thicker by DIC optics, and more complex than lamellae in E20, P5, or P5^{KLF4} growth cones, protruding many small filopodia during lamellar advance (Fig. 2B), which is consistent with closely linked lamellar protrusion

FIGURE 2. In P5 RGCs, the axon growth suppressor KLF4 (P5^{KLF4}) and the axon growth enhancer KLF6 (P5^{KLF6}) differentially regulate lamellar and filopodial dynamics. Time-lapse sequences show that (A) P5^{KLF4} protruded few, small lamellae (L) and numerous, long filopodia, whereas (B) P5^{KLF6} also elaborated numerous filopodia and few but much larger lamellae. Scale bar: 5 μm. (C) Axon growth rate was enhanced 4-fold in P5^{KLF6}, approaching E20 rates. Normalized to E20 (dashed reference line), (D) lamellar initiation frequency remained low for both KLFs. In P5^{KLF6}, lamellar area was greater than in P5^{KLF4} and lamellar distance increased almost to E20 distances, although lamellar protrusion rates were similar to E20 for both KLFs. (E) Filopodial initiation frequencies and protrusion rates were similar to E20 for both KLFs. Both KLFs increased filopodial binding to the substrate but to a lesser extent in P5^{KLF6}. In P5^{KLF6}, but not in P5^{KLF4}, filopodial rigidity increased almost to E20 levels. Filopodial lifetime was greater in P5^{KLF4}, whereas in P5^{KLF6}, lifetimes were closer to E20. Non-normalized means are indicated above each bar. Significance was calculated between P5^{KLF4} and P5^{KLF6}. **P* < 0.001, ***P* < 0.05, *n* = 3 per condition.

and engorgement. In these experiments, P5^{KLF4} axons grew at only one-fifth the rate of P5^{KLF6} axons, which grew approximately 6-fold faster than P5 axons (9.3 μm/h), nearly up to E20 axon growth rates (Fig. 2C). Previously, we showed that KLF4 overexpression reduced long-term postnatal axon growth rates measured over 3 days in vitro.¹⁹ However, in this study, KLF4 overexpression failed to decrease short-term axon growth rates in P5 RGCs. Therefore, short-term axon growth rates are insufficient by themselves to explain KLF4-mediated long-term reductions in postnatal RGC axon growth.

Lamellar protrusion dynamics were also differentially regulated in P5 RGCs by KLF4 and KLF6 overexpression (Fig. 2D). Both lamellar area and distance differed. In P5^{KLF4}, the mean lamellar area was significantly smaller compared with the mean lamellar area in P5^{KLF6}, which was approximately 60% of E20. In P5^{KLF4}, lamellae protruded 4.8 μm, similar to P5 (5.3 μm), whereas P5^{KLF6} lamellae protruded 6.5 μm, almost to E20 distances (Fig. 2D). However, similar to both E20 and P5, both P5^{KLF4} and P5^{KLF6} protruded lamellae at similar rates. Thus, neither lamellar initiation frequency nor lamellar protrusion rate explained the developmental differences in axon growth rate; increased axon growth rate in P5^{KLF6} is characterized by increased lamellar distance and area, mimicking the differences in lamellar dynamics between E20 and P5 RGCs.

Filopodial dynamics also were differentially regulated in P5 RGCs by KLF4 and KLF6 (Fig. 2E). Quantitative analysis of both P5^{KLF4} and P5^{KLF6} growth cones revealed that compared with E20 RGCs, both P5^{KLF4} and P5^{KLF6} lamellae initiated similarly at low frequencies. The mean filopodial protrusion rates in P5^{KLF4} and P5^{KLF6} were similar to E20 filopodial initiation rates. In all other measures of filopodial dynamics, P5^{KLF6} growth cones

more closely mimicked E20 growth cones: P5^{KLF6} growth cones protruded filopodia that were shorter-lived, spent less time bound to the substrate, and largely retained their rigidity, similar to E20 RGCs and consistent with the observed increase in lamellar protrusion area in P5^{KLF6} (Fig. 2E).

To determine if lamellar or filopodial dynamics predict short-term axon growth rates, the linear relationship between axon growth rate and each measured lamellar or filopodial characteristic was determined by calculating Pearson correlation coefficients (Fig. 3A). Several lamellar and filopodial dynamics correlated significantly with axon rate, including lamellar initiation, distance, and area, and filopodial lifetime, time bound to the substrate, and rigidity. To determine which, if any, of these dynamics explained short-term axon growth rates, all variables were entered into a forward stepwise linear regression model. Only one variable entered, lamellar area (*P* < 0.001), which explained 79% of the variability in axon rate. Axon rate was plotted against lamellar area by group (Fig. 3B). The E20 had the highest values for both lamellar area and axon rate, P5^{KLF6} demonstrated an intermediate level of lamellar area and axon rate, and P5 and P5^{KLF4} had the lowest values.

What features of growth cone dynamics best predicted lamellar protrusion area? Three related filopodial characteristics, lifetime, time bound to the substrate, and the percentage of time bound that filopodia remained rigid, correlated significantly with lamellar area (Fig. 3A). Multivariate regression analysis revealed that filopodial rigidity explained 68% of lamellar protrusion area (*P* < 0.001), consistent with filopodial adhesion-based signaling disrupting not only filopodial integrity³⁵ but also inhibiting lamellar protrusion on laminin.²⁵

FIGURE 3. Lamellar area correlates most strongly with axon growth rate. **(A)** Pearson coefficient table for neurite rate and all measured lamellar and filopodial dynamics. Shaded boxes indicate significance ($P < 0.05$, $n = 12$). **(B)** Lamellar area most strongly correlates with neurite rate. Plot of neurite rate versus lamellar area for E20 (open), P5 (black), P5^{KLF4} (light gray), and P5^{KLF6} (dark gray). $n = 12$ per condition.

Postnatal Axon Growth Is Slowed by Increased Inactive Growth States

We next asked whether the difference in short-term axon growth rates and growth cone dynamics characterized above correlated with differences in the growth cone's instantaneous growth rate, time spent paused (motile but without forward or backward movement), or collapsed. We analyzed mean and instantaneous axon growth rates and the time spent in an active or collapsed morphology in time-lapse recordings (Figs. 4A, 4B). In these experiments, the mean axon growth rate, over the total 60- to 240-minute recordings, was 4-fold higher in E20 (0.8 $\mu\text{m}/\text{min}$) compared with P8 (0.2 $\mu\text{m}/\text{min}$; Fig. 4C), consistent with previous analyses. However, despite different short-term growth rates, the times E20 and P8 axons spent growing at different growth rates, disregarding periods of pause, collapse, or retraction were similarly distributed (Fig. 4D). During active growth cone advance, the mean axon growth rate for E20 axons was 1.1 μm per minute only 1.5-fold faster than P8 axons at 0.75 μm per minute. In contrast, analysis of the relative contributions of active advance, time spent paused, and time spent collapsed with or without retraction, to overall axon growth rate revealed the majority, 72%, of decreased P8 axon growth rate was explained by increased time the growth cone was motile but paused (Fig. 4E). The remainder was largely explained by longer retraction phases in P8 RGC axons (not shown). Thus, the decline in postnatal RGCs' intrinsic capacity for rapid axon growth is due

FIGURE 4. Decreased intrinsic axon growth rate in postnatal RGCs is attributable to growth cone pausing. **(A, B)** Axon growth rate traces of E20 and P8 RGCs in culture. **(C)** The mean axon growth rate was approximately 4-fold higher in E20 (black) compared with P8 (gray) axons. **(D)** Histogram of time spent growing at different rates during active growth states only. Mean growth rates during active elongation for E20 (black arrow) and P8 (gray arrow) are indicated. **(E)** E20 growth cones spent more time in an active growth state, extending, whereas P8 growth cones spent more time paused. $n \geq 7$ axons per age. $*P < 0.001$. Error bars indicate SEM.

FIGURE 5. External tension supports increased axon growth rates. (A, B) A postnatal growth cone (GC) advancing on laminin was attached to a laminin-coated glass needle and its growth cone elevated immediately above the culture dish. (C) Tension applied to the elevated growth cone led to axon elongation over 80 μm , after which the growth cone was placed back on the laminin substrate (solid arrow). (D) β -III-tubulin polymer labeling revealed the stretched axon filled with microtubules. Note, the axon continued to elongate after reattaching to the laminin substrate (compare GC to solid arrow in [D]). Also, the stretched axon labeled more intensely than an unstretched axon (dashed arrow) from the same cell. Scale bar: 20 μm for (A–D). (E) Applying mechanical tension increased neurite growth up to 15 μm per minute similarly in both embryonic (solid symbols, solid linear trendlines) and postnatal axons (hollow symbols or stars, dashed linear trendlines).

both to growth cone pausing and retraction, as well as to a slightly decreased ability to achieve rapid instantaneous rates of forward progression.

The postnatal failure to sustain forward progression could be due to a failure to adequately supply the building blocks for elongation, or to a growth cone mechanism independent of cellular resources. To determine if RGCs' decreased postnatal growth state is due to a lack of cellular resources independent of the growth cone, growth cone-mediated elongation was bypassed by allowing growth cones to attach to a laminin-coated glass needle (Figs. 5A, 5B) and then mechanically applying 50 to 250 μD of tension force. Consistent with previous studies,^{32,33} RGC axons could be elongated and then placed back on the laminin substrate (Fig. 5C). Subsequently, the growth cones of stretched RGC axons continued to advance on laminin, further elongating the axon and indicating that elongation by mechanical tension does not disrupt the capacity for growth cone-mediated advance (Fig. 4D). Anti- β -III-tubulin filament labeling, after membrane extraction and monomer washout, revealed that tension-elongated axons filled with microtubules to a similar or to a greater extent than unstretched axons (Fig. 5D), consistent with growth by tension-induced mass addition along the tension-elongated RGC axon.³⁶ With increasing tension, axon growth rate could be increased up to 900 μm per hour (Fig. 4E), approximately 100-fold faster than normal postnatal RGCs (9.3 $\mu\text{m}/\text{h}$). Together, these results show that postnatal RGCs have sufficient cellular resources to support rapid axon growth rates and further support the hypothesis that

FIGURE 6. Intrinsic axon and dendrite growth in RGCs are independently regulated. Scatterplots of total axon and total dendrite lengths of RGCs purified from E19 (A) and P8 (B) rats; each diamond represents the measurements from a single RGC. Regression analysis revealed axon and dendrite growth are regulated independently at both ages. $n = 46$ RGCs per age.

postnatal axon growth rate is limited by mechanistic changes at the growth cone.

Growth-Specific Programs Regulate Axon and Dendrite Growth Independently

We previously found that during early postnatal development, RGCs turn off their intrinsic axon growth ability and, through the same time period, increase their intrinsic dendrite growth ability.² To assess to what degree the decline in axon growth ability is co-regulated with or by the increase in dendrite growth ability and to determine if this shift to dendritic growth limits axonal growth due to shifting limited cellular resources, we examined the correlation of axon and dendrite growth within embryonic and postnatal RGC populations. Under a “conserved resource” hypothesis, axonal growth would be suppressed by shifting limited cellular resources away from axons to support dendrite growth, and axon and dendritic growth would be inversely correlated. Alternatively, under a “global neurite growth capacity” hypothesis, both axon and dendrite growth capacity would be specific to individual neurons and positively correlated. The third possibility would be an “independent regulation” hypothesis, in which case axon and dendrite growth would demonstrate no correlation. Regression analysis revealed axon and dendrite growth were neither inversely nor positively correlated in both embryonic (Fig. 6A) and postnatal RGCs (Fig. 6B). Therefore, neither limited cellular resources nor a set cellular growth capacity explains decreased axon growth in postnatal RGCs, supporting the hypothesis that an axon-specific growth program regulates axon growth, and dendrite growth is regulated independently.

DISCUSSION

Together, these data show intrinsic, short-term, axon growth is regulated at the growth cone by an axon-specific growth program that regulates age-dependent changes in the axonal growth cone's protrusive activity. Our finding that postnatal RGC axons can support axon elongation rates 100-fold greater than growth cone-directed elongation rates and that the relationship between induced tension and elongation rate is linear, are consistent with similar data from peripheral neuron^{32,37} and embryonic CNS neurons³³ and with the rapid axon growth observed during development, which is largely due to passive stretching as the organism grows.³⁸ Here, directly comparing embryonic to postnatal neurons, we found that although growth cone-induced elongation is slower in postnatal than in embryonic RGCs, both could support similar elongation rates with externally applied tension. This was evidenced on a short time scale, reducing the likelihood that the tension increased production of cellular resources through

distant (e.g., nuclear/cell body) transcription or translation. Thus, axon growth potential is limited by local, growth cone-related dynamics, but not on the supply of cellular resources. Furthermore, the tension-elongation rate relationship was in the same range at both ages, suggesting the basal cellular capacity to support axon elongation is not fundamentally different between the two ages.

At the growth cone, increased short-term axon growth rates were largely explained by the area of lamellar protrusions in both embryonic RGCs and postnatal RGCs expressing exogenous KLF6, consistent with other studies in rat cervical ganglion cells, showing that primarily lamellar growth cones support faster growth than more filopodial growth cones.³⁹ Although increased lamellar area may be a downstream consequence of another effector regulating rapid axon growth, rather than a proximate cause, as data support the converse hypothesis, differences in lamellar protrusion regulate axon growth, with more lamellar growth cones supporting more rapid growth both *in vitro*^{39,40} and *in vivo*.^{41,42} Lamellae provide conduits for engorging microtubules and vesicles to advance the growth cone and to elongate the neurite,⁴³ and may also regulate axon growth by differentially regulating the detection and signal transduction of extracellular cues.⁴⁴ Thus, larger lamellae may promote axon growth by simply providing more space for microtubule and vesicular engorgement.⁴⁵ However, lamellae also can slow axon growth,⁴⁶ axons can grow rapidly with little lamellar protrusion *in vitro*⁴⁷ and *in vivo*,⁴⁸ and in some cases, filopodial growth cones grow faster than more lamellar growth cones.⁴⁹ Thus, increasing intrinsic growth rate by increasing lamellar protrusion area likely requires the cooperation between multiple signaling factors, including the growth cone's receptor repertoire and cytoskeletal and adhesion dynamics. Thus, determining how altering intrinsic axon growth states, for example, by manipulating transcription factor KLFs, regulates growth cone receptor distribution, cue detection and transduction, and guidance, will be important for understanding how axons grow during development and for promoting successful regeneration in the adult.

How do intrinsic growth states differentially regulate lamellae? Despite significant differences in several lamellar and filopodial dynamics, the rate lamellae protruded remained constant among all four groups analyzed, indicating differences in lamellar protrusion distance and area are not due to differences in the absolute rate of filamentous actin (F-actin) polymerization at the leading edge. Lamellar protrusion is regulated by a balance between F-actin polymerization at the leading edge in the peripheral zone and F-actin retrograde flow⁵⁰ and de-polymerization in the growth cone's transition zone between the peripheral and central domains.⁵¹ Thus, the smaller lamellar protrusions in postnatal RGCs and in postnatal RGCs expressing KLF4 are likely due to differences in the distribution and activities of the large number of molecules regulating actin filament polymerization and dynamics, such as myosin II, which primarily drives retrograde flow,⁵² cofilin, which regulates actin turnover,^{53,54} and/or the opposing activities of F-actin filament capping and uncapping proteins like Ena/VASP family members.⁵⁵ The large number of changes in growth cone dynamics we observed suggests a model that may provide a direct means for testing how different actin filament regulators are differentially regulated by different intrinsic growth states.

What differences in lamellar and filopodial morphology, dynamics, or signaling could explain the observation that the decrease in postnatal axon growth rate is largely due to growth cone pausing? The molecular pathways that regulate axon growth rates may have more to do with actin cycling dynamics, versus those that regulate the growth cone's decision to

elongate from a paused position, which may have more to do with the engagement of cycling actin by a molecular "clutch" signaled by external cues. Our observation that embryonic filopodia retained their rigidity, spent little time bound to the substrate and were short-lived, whereas postnatal filopodia failed to maintain their structural integrity, remained bound to the substrate, were long-lived, and failed to support lamellar protrusion, are consistent with increased filopodial adhesions on laminin reducing filopodial motility⁵⁶ and restricting both lamellar protrusion²⁵ and neurite growth.³⁵

Together these data support the hypothesis that differences in lamellar and filopodial signaling suppress postnatal axon growth by restricting active growth states; these data may guide future study of the molecular regulation of growth cone dynamics and axon elongation. Indeed, increased time in an inactive or paused growth state is implicated in failed regeneration in the CNS.⁵⁷ These pauses are characterized by changes in actin^{58,59} and microtubule dynamics,^{60–62} as well as local changes in protein¹⁷ and lipid synthesis.⁶³ In particular, the neuronal ubiquitin ligase, PHR, is hypothesized to regulate microtubule disassembly and the ability of growth cones to exit paused states at intermediate targets.⁶⁴ The data in this article are limited by their correlative nature, and to directly test such hypotheses in RGCs, future work could directly manipulate growth cone lamellipodia or filopodia structures by expressing cytoskeletal proteins that specifically regulate these structures in the growth cone (e.g., Ena/VASP, Rac, Arp2/3). Analyzing how these and other factors change with intrinsic changes in RGC growth cone dynamics and elongation may help to identify effectors for promoting intrinsic axon growth capacity in CNS neurons.

An important implication of this research concerns the cross regulation of axon and dendrite growth in neurons. The premise that there should be a co-dependence of axons and dendrites on the same general pool of cellular growth resources is consistent with the hypothesis that competition between axons and dendrites regulates neurite growth.^{65–67} However, our results argue against this hypothesis, because axon and dendrite growth were regulated independently, in both embryonic and postnatal RGCs. Other studies also have shown that the fast growth of one neurite is not compensated by slower growth or retraction of other neurites, even under the increased growth rates seen under applied mechanical tension.⁶⁸ Thus, our data support a model in which different programs regulate the developmental increase in axon and decrease in dendrite growth ability. This may prove important, as effects on dendritic elongation and plasticity are rarely studied in experiments manipulating axon regeneration. One recent study examined the effects of neurotrophic factors, for example, on RGC dendrites after optic nerve injury, and found that AAV vector-mediated expression of neurotrophic factors, including BDNF and CNTF, had measurable, gene-specific effects on dendritic morphology in injured adult RGCs.⁶⁹ Identifying the extrinsic factor(s) regulating these shifts in dendrite growth may prove important to understanding and ultimately providing new strategies for promoting repair *in vivo* after injury, by regulating specific axon or dendrite growth programs to elicit long-distance regeneration or local plasticity after injury or neurodegenerative disease.

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