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Activation of PI3K and R-Ras Signaling Promotes the Extension of Sensory Axons on Inhibitory Chondroitin Sulfate Proteoglycans

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Abstract

Chondroitin sulfate proteoglycans (CSPGs) are extracellular inhibitors of axon extension and plasticity, and cause growth cones to exhibit dystrophic behaviors. Phosphoinositide 3-kinase (PI3K) is a lipid kinase activated by axon growth promoting signals. In this study we used embryonic chicken dorsal root ganglion neurons to determine if CSPGs impair signaling through PI3K. We report that CSPGs inhibit PI3K signaling in axons and growth cones, as evidenced by decreased levels of phosphorylated downstream kinases (Akt and S6). Direct activation of PI3K signaling, using a cell permeable phospho-peptide (PI3Kpep), countered the effects of CSPGs on growth cones and axon extension. Both overnight and acute treatment with PI3Kpep promoted axon extension on CSPG-coated substrates. The R-Ras GTPase is an upstream positive regulator of PI3K signaling. Expression of constitutively active R-Ras promoted axon extension and growth cone elaboration on CSPGs and permissive substrata. In contrast, an N-terminus-deleted constitutively active R-Ras, deficient in PI3K activation, promoted axon extension but not growth cone elaboration on CSPGs and permissive substrata. These data indicate that activation of R-Ras-PI3K signaling may be a viable approach for manipulating axon extension on CSPGs.

INTRODUCTION

Development of the nervous system is dependent upon complex interactions between neurons and their environment. The extension and guidance of neuronal axons is of primary importance in the establishment of proper patterns of neuronal circuitry. The growth cone, a dynamic protrusive structure localized at the tip of developing axons, is responsible for both the extension and guidance of axons (Dent and Gertler, 2003). The actin filament-dependent

dynamics of growth cones are a major determinant of axon guidance and extension. The de novo formation of axon branches from the shaft of the axon, generated behind the advancing growth cone, is also a fundamental aspect of neurodevelopment (Gibson and Ma, 2011). Axon branching allows a single axon to establish synaptic contacts with multiple target cells and central nervous system domains. Similar to the growth cone, the formation of axon branches is also dependent on actin filament dynamics (Gallo, 2011).

Chondroitin sulfate proteoglycans (CSPGs) are a class of extra-cellular matrix molecules with a variety of functions in both the developing and adult nervous system (Bartus et al., 2012; Kwok et al., 2012; Cregg et al., 2014). Following the establishment of the basic patterns of neuronal connectivity, CSPGs have roles in the maintenance and stability of circuitry through the establishment of peri-neuronal nets (Pizzorusso et al., 2002). In the adult nervous system, CSPG expression occurs in regions of injury, and inhibits axon regeneration and sprouting of collateral branches (Bartus et al., 2012). Recent studies have identified receptor systems that mediate the effects of CSPGs on developing and regenerating axons which include LAR, PTP σ and Nogo receptors (Coles et al., 2011; Fisher et al., 2011; Sharma et al., 2012). However, much remains to be learned about the signaling mechanisms that mediate the effects of CSPGs on axons and growth cones.

Phosphoinositide 3-kinase (PI3K) signaling is a major regulator of axon development (Waite and Eickholt, 2010), and Ras GTPases are upstream activators of the PI3K pathway (Goldfinger, 2008). PI3K is a lipid kinase that initiates an intracellular signaling cascade mediated by Akt/PKB, which can regulate cytoskeletal dynamics, cellular bioenergetics, gene transcription and mRNA translation (Cosker and Eickholt, 2007). Many growth factors, including nerve growth factor (NGF), that positively regulate axon extension and branching activate PI3K signaling (Patapoutian and Reichardt, 2001; Gallo, 2011; Mack and Eickholt, 2011). NGF activates multiple pathways, including R-Ras signaling which in turn is a potent driver of PI3K activity (Rodriguez-Viciano et al., 2004; Iwashita et al., 2007; Marte et al., 1997). In contrast, some extracellular signals that inhibit axon extension act, at least in part, through suppression of PI3K signaling (Atwal et al., 2003; Chadborn et al., 2006; Gallo, 2008; Oinuma et al., 2010; Fisher et al., 2011). In this paper, we present evidence that chicken nervous system CSPGs inhibit the extension and growth cone dynamics of embryonic chicken dorsal root ganglion sensory neuron axons through inhibition of PI3K signaling, and that experimental activation of R-Ras-PI3K signaling reverses the effects of CSPGs on axons and growth cones.

METHODS

Culturing of sensory neurons and homogeneous substratum preparation

Chicken embryonic dorsal root ganglion explants (E7-14) and dissociated neurons (E7) were cultured using standard protocols in defined F12H medium (Lelkes et al., 2006).

Experiments addressing the effects of NGF were performed with E7 neurons, as at this developmental stage neurons extend axons in the absence of NGF allowing for studies of the effects of acute treatment with NGF. Experiments involving PI3K pep /PI3K pepAla were performed using E14 explants, while experiments involving R-Ras were performed using E7 neurons. E7 neurons were used for transfection experiments as neurons of this age exhibit

better electroporation mediated transfection and survival than E14. Laminin (Invitrogen) was used at 25 µg/mL for standard culturing conditions or 15 µg/mL for background coating in conjunction with CSPGs. Poly-d-lysine (70–100 KD, Sigma) was used at 100 µg/mL and dissolved in borate buffer. Embryonic day E12–14 chicken brain CSPGs (Millipore) were resuspended in deionized water and used at the described concentrations. For preparation of CSPG substrata, polylysine was first applied, followed by CSPGs and finally laminin, with washes of deionized water between coating steps. For experiments involving fixed samples, cultures were made on glass coverslips. For experiments requiring live imaging, cultures were prepared on glass coverslip bottomed “video dishes” as previously described (Spillane et al, 2011).

Preparation of substrata borders and analysis of axon turning

Borders between a permissive substratum (polylysine+laminin) and a CSPG inhibitory substratum (CSPGs+polylysine+laminin) were generated as previously described (Ketschek et al., 2012). Briefly, the coverslips were coated with 100 µg/mL of poly-D-lysine (70 KD MW; Sigma) overnight. The following day, the coverslips were washed with deionized water and air dried. A 1 mm thick strip of filter paper (Fisherbrand P5) saturated with 3 µL of 100 µg/mL CSPGs was applied to the coverslip using fine forceps. Once the filter paper appeared dry, it was removed from the coverslip. The coverslip was then washed and coated with 15 µg/mL laminin for 1 hr at 37° C prior to use. The turning of axons at borders between the permissive and CSPG substrata was performed as in Ketschek et al (2012).

Culturing of NIH3T3 cells and western analysis

NIH3T3 fibroblasts were grown in DMEM/10% bovine calf serum, transfected with GFP-R-Ras fusion plasmids (Wurtzel et al., 2012), serum starved and lysed as described. Lysates were separated by SDS-PAGE and probed with antibodies to Akt and anti-phospho-Ser473-Akt as described. GFP-DN-R-Ras(G38V) was made by deletion of the N-terminal 26 amino acid coding sequence from the GFP-R-Ras(G38V) plasmid. GFP fusion of the isolated R-Ras N-terminus (N-term) was created by insertion of the N-terminal 26 amino acid coding sequence in the pEGFP vector (Clontech). Western analysis and quantification of pAkt and total Akt levels were performed as described in Wurtzel et al (2012).

Transfection and peptides

Transfection of dissociated neuron cultures was performed as previously described (Spillane et al., 2011). Briefly, dissociated neurons (40 ganglia/transfection) were transfected using an Amaxa Nucleofector electroporator (Amaxa Inc) and chicken neuron-specific transfection solutions (Amaxa Inc). We routinely used 5–10 µg of plasmid DNA per transfection. For co-transfections, both plasmids were added together prior to electroporation at sufficiently high concentrations so that the final volume of plasmid solution was not greater than 15 µL. Neurons were plated immediately following electroporation. GFP plasmid was obtained from the Amaxa transfection kits. mCherry plasmid was as described in Spillane et al (2012). NIH3T3 cells were transfected using Lipofectamine (Invitrogen) as described (Wurtzel et al., 2012).

PI3Kpep and PI3KpepAla were custom synthesized by American Peptide Company, and used as in our previous work (Ketschek et al., 2010; Spillane et al., 2011, 2012). The peptides are rendered cell permeable by being synthesized in tandem with the antennapedia cell permeable peptide sequence at the N-terminus. Peptide stocks were resuspended in F12H medium, water bath sonicated to break aggregates, aliquoted and stored at -80°C at a stock concentration of 1 mg/mL in culturing medium (NGF was added to peptide-containing medium prior to treatment of cultures). Peptides were thawed at room temperature, water bath sonicated for 10 min to break up clusters, and filtered through a 0.2 μm syringe filter prior to use. A routine final concentration of 50 $\mu\text{g/mL}$ of peptides was used based on our previous work with these peptides.

Immunocytochemistry

For antibody staining, cultures were fixed with 4% paraformaldehyde in PBS containing 5% for 15 minutes. Following washing, blocking solution was applied for 30 min (10% goat serum in PBS + 0.1% Triton X-100), followed by staining with blocking solution containing the primary antibody for 1 hr at room temperature, washing again, and application of the secondary antibody in blocking solution. Cultures were then washed again and stained with rhodamine phalloidin, when described as such in the results. The primary antibodies and the dilutions they were used at were as follows: anti-phospho-Ser473 Akt (Cell Signaling, 1:200), anti-phospho-Ser235/236 S6 (Cell Signaling, 1:200), anti-R-Ras (Santa Cruz, 1:400). CSPG substrata were stained with anti-CSPG CS-56 (Sigma, 1:500), as in Ketschek et al (2012). Tubulin and actin filaments were stained using anti- α -tubulin DM1A-FITC (1:100, Sigma) and rhodamine phalloidin following the manufacturer's protocol (Invitrogen), respectively. All secondary antibodies were obtained from Jackson ImmunoResearch.

Quantitative pAkt and pS6 immunocytochemistry

For quantitative analysis of antibody staining intensity all relevant experimental groups were processed in parallel using the same solutions for all coverslips. Images from relevant samples were then acquired using identical image acquisition settings during the same microscopy session. Acquisition parameters were set such that the entire data sets were within the dynamic range of measurement. Imaging was performed on a Zeiss 200M inverted microscope equipped with an Orca ER camera (Hamamatsu) using Zeiss Axiovision software. Images were acquired at 2 $\times$ 2 binning and analyzed using the measurement module of Axiovision software. A fixed 50 μm length of the distal axon, including the growth cone, was outlined as a region of interest. The mean intensity of antibody staining was then determined within the region of interest for each axon sampled.

Morphometrics and live imaging

All morphometric image analysis was performed using the measurement module of Zeiss Axiovision software. In dissociated neurons, axon lengths were determined by tracing the length of the axon from its hillock at the cell body to the center of the growth cone (40 $\times$ images). In experiments investigating the effects of CSPGs on axon length, only neurons with axons greater than 30 μm were measured. This criterion was adopted based on the observation that on CSPGs neuronal cell bodies either exhibited no processes, although they

formed small lamellipodia and filopodia from the cell bodies, exhibited stumpy processes/axons less than 30 μm , or axons that extended significantly longer than 30 μm . Upon dissociation and plating, a proportion of sensory neurons have stumpy processes up to 30 μm in length, and thus we feel that processes in this length range are best not considered in measurements of axon length as they may not have extended from the cell bodies *de novo*. These morphological criteria were also used for determination of overall process/axon extension on CSPGs. In experiments using dissociated ganglia, only axons that did not contact other cells (ganglionic satellite cells, fibroblasts or other neurons as imaged through phase optics or counter staining with phalloidin) were sampled to exclude confounds from cell-cell contact. Growth cone filopodial number was determined by counting the number of filopodial tips present along the distal 20 μm of axons, representing the growth cone and 10–15 μm of the distal axon shaft. Growth cone area was measured from a region of interest outlining the perimeter of the growth cone excluding filopodia. The most proximal growth cone was defined as the point where the axon shaft visibly spread out. If the axon tip did not spread out (i.e., the growth cone did not exhibit lamellipodia) then the area measurement was performed on the distal 10 μm of the axon.

Live imaging of axon extension was performed using the same microscopic set up described above. Imaging was performed using 20x phase contrast objectives and 1×1 camera binning. Images were acquired every 3 minutes. Temperature was kept constant at 39° C using a Zeiss 3-plate stage heating insert. Our culturing medium, F12H, is HEPES-buffered and does not require CO₂ chambers. Measurement of axon extension was determined by segmented line analysis connecting the position of the center of the growth cone at time zero to that at the end of the video sequence. For peptide treatments, the video sequence was commenced immediately following treatment.

Statistics

All statistical analysis was performed using InStat 3.10 (GraphPad Inc). The software automatically checks for normalcy in data sets. For normally distributed data sets, analysis was performed using parametric Welch *t*-tests not using assumptions about equal standard deviations. For non-parametric data, the Mann-Whitney test was used. In multiple comparison scenarios, post-hoc tests were used. Categorical data sets were analyzed using the Fisher's exact test run on raw numerical data (although percentages are used for data presentation) or the χ^2 test for independence for 3 or more categories.

RESULTS

CSPGs inhibit PI3K signaling in axons

CSPGs inhibit the extension of chicken sensory axons (Ketschek et al., 2012). In order to determine suitable concentrations of CSPGs for the remainder of our studies we first determined the concentration-dependent effects of CSPG-coated substrata on axon extension from cultured dorsal root ganglion explants. Compared to control polylysine + laminin coated substrata, further coating of the substrata with concentrations of CSPGs from 10–100 $\mu\text{g/mL}$ decreased axons lengths (not shown). The effect was strongest at 50–100 $\mu\text{g/mL}$, resulting in approximately a 60% decrease in mean axon length. However, at 50 $\mu\text{g/mL}$ we

observed more axons extended from explants. The axons of embryonic day (E) 7 to E14 responded similarly to CSPGs (not shown). The 50 µg/mL dose was thus chosen in order to balance a strong inhibitory effect with sufficient axons extending from explants for sampling purposes, unless otherwise indicated.

PI3K activity drives the phosphorylation of the downstream kinase Akt, resulting in its activation, and in turn activated Akt drives the downstream phosphorylation of ribosomal subunit S6 by mTOR (Crino, 2011). Nerve growth factor (NGF) activates PI3K signaling in sensory neurons, and PI3K signaling is required for NGF-induced sensory axon extension, growth cone dynamics, axon collateral branching and intra-axonal protein synthesis (Ketschek et al., 2010; Spillane et al., 2011, 2012). In order to address the feasibility of using quantitative immunocytochemical approaches to determine the extent of PI3K signaling directly in axons, we determined the levels of staining with antibodies against phosphorylated Akt (Ser473; pAkt) and S6 (Ser235/236; pS6). Embryonic day 7 chicken dorsal root ganglion explants were cultured overnight on a laminin substratum in the absence of NGF, conditions which allow NGF-independent outgrowth of NGF-responsive neurons, and thus investigation of the effects of acute NGF treatment (Ketschek et al., 2010; Spillane et al., 2011, 2012). Cultures were then treated with NGF, fixed and stained with relevant antibodies. NGF treatment increased the staining levels of pAkt and pS6 in axons approximately 2- and 2.5-fold, respectively (Figure 1A–C), indicating that this quantitative immunocytochemical approach reliably detects changes in PI3K activity in axons.

Fischer et al (2011) previously reported that CSPGs decrease the total levels of Ser473 pAkt by approximately 40% in mass cultures of cerebellar granule neurons, through western blotting. In order to determine directly if CSPGs affect pAkt levels in axons, we used the quantitative immunocytochemical approach described above to determine the levels of pAkt and pS6 in E14 axons. Comparison of the levels of pAkt and pS6 in the axons of neurons on substrates also coated with CSPGs relative to those on polylysine + laminin alone, but equally cultured in the presence of NGF, revealed that PI3K signaling was markedly decreased by CSPGs locally in distal axons (Figure 1C). These observations confirm and extend the work by Fischer et al (2011) by also revealing suppression of pS6 levels (Figure 1C).

Direct activation of PI3K using a cell permeable peptide (PI3Kpep) increases the levels of pAkt and pS6 in axons growing on CSPGs

PI3Kpep is a previously described cell permeable phospho-peptide which directly activates PI3K (Williams and Doherty, 1999; Chadborn et al., 2006; Gallo, 2008; Ketschek et al., 2010; Spillane et al., 2011, 2012), and we have previously reported that it mimics the effects of NGF on axon branching, axonal actin filament dynamics, and NGF-induced intra-axonal protein synthesis (Ketschek and Gallo, 2010; Spillane et al., 2011, 2012). As in our previous work, the control for PI3Kpep is PI3KpepAla. PI3KpepAla lacks a crucial phospho-tyrosine which is replaced by an alanine. We used quantitative immunocytochemistry, as described above, to determine if PI3Kpep treatment would promote pAkt and pS6 levels in axons growing on CSPGs. Explants of E14 sensory ganglia were cultured on polylysine + laminin with or without further coating with CSPGs, and cultured in the continuous presence of

NGF. A 1 hr treatment with a concentration of PI3Kpep that maximally affects axon morphology (50 µg/mL) restored the levels of pAkt in axons seeded on CSPG substrata to levels analogous to those observed in the absence of CSPGs, whereas PI3KpepAla treatment did not restore the levels of pAkt in the presence of CSPGs (Figure 2). Similarly, PI3Kpep, but not PI3KpepAla, elevated the levels of pS6 in axons on CSPGs, but unexpectedly increased them above baseline control levels (Figure 2).

Treatment with PI3Kpep restores growth cone morphologies on CSPG substrata and promotes axon crossing at borders between CSPGs and a permissive substratum

CSPGs affect growth cone morphology, characterized by a decrease in the protrusive activity of lamellipodia and filopodia, resulting in dystrophic growth cones (Tom et al., 2004; Beller et al., 2013). In order to track the effects of CSPGs on growth cone morphology, we quantified growth cone area, reporting on lamellipodial protrusions and the number of filopodia along the axons of E14 sensory neurons. Compared to baseline culturing conditions (polylysine + laminin + NGF + 1 hr treatment with PI3KpepAla), the growth cones of axons treated for 1 hr with PI3KpepAla extending on CSPGs exhibited decreased area and number of filopodia (Figure 3A, B). A 1 hr treatment with PI3Kpep restored growth cone area and the number of filopodia to baseline levels in axons growing on CSPGs (Figure 3A, B).

CSPGs can act as growth cone repellents, inhibiting axon regeneration in the injured spinal cord (Kwok et al., 2012). In this scenario, regenerating axons encounter a domain of high CSPG expression at the injury site. To determine if PI3Kpep treatment would allow axons to cross from a permissive substratum (polylysine + laminin) onto a CSPG substratum (polylysine + laminin + CSPGs), we generated borders between these two substrata as in our previous work (Ketschek et al., 2012) based on the protocols of Challacombe et al. (1997) and Beller et al. (2013). As expected, in the presence of PI3KpepAla, axons failed to cross from the permissive substratum onto CSPGs. In contrast, treatment with PI3Kpep increased the proportion of axons that crossed onto the CSPGs substratum by approximately 4 fold (Figure 3C, D).

Treatment with PI3Kpep promotes axon extension on CSPG substrata

We cultured dorsal root ganglion explants overnight on CSPG-coated substrata in the presence of PI3Kpep or PI3KpepAla, and NGF. PI3Kpep increased the density and length of axons extending from the explants (Figure 4A). Relative to PI3KpepAla, PI3Kpep treatment increased the number of axons that crossed circumferential lines drawn at 300 and 400 microns from the base of the explant (Figure 4B). In order to determine directly if PI3Kpep treatment promotes the rate of axon extension on CSPG substrata, we cultured sensory explants on CSPG-coated substrata and determined the effects of PI3Kpep treatment on axon extension and growth cone dynamics through phase contrast imaging. Axons were imaged using phase contrast imaging for 90 min starting immediately following addition of peptides to the cultures. Direct measurement of the rate of axon extension following treatment with PI3Kpep or PI3KpepAla on CSPG-coated substrata revealed that PI3Kpep treatment increased the mean rate of axon extension by 3 fold relative to PI3KpepAla treatment (Figure 4C, D). Following treatment with PI3KpepAla, 10/29 axons exhibited no

growth, compared to 1/18 with PI3Kpep treatment, and 2/29 axons underwent net retraction, in contrast to none in the PI3Kpep treatment group. Consideration of the rates of axon extension during the first and subsequent 9 min of treatment (0–18 min) with PI3Kpep or PI3KpepAla revealed that PI3Kpep increases axon extension after the first 9 min of treatment (Figure 4E). Furthermore, during the initial 18 min of treatment 7/29 of PI3KpepAla treated axons underwent retraction during one of the 9 min periods, compared to none in the PI3Kpep treated group. Although during the first 0–9 min of treatment with PI3Kpep the mean rate of axon extension ($6.8 \pm 1.8 \mu\text{m}/9 \text{ min}$, mean SEM) was increased relative to PI3KpepAla treatment ($1.8 \pm 0.7 \mu\text{m}/9 \text{ min}$), this did not reach significance. Comparison of the rate of axon extension following PI3Kpep treatment at 0–9 and 9–18 min showed a 58% increase during 9–18 min relative to 0–9 min ($p < 0.04$, Paired t-test). These data indicate that the effects of PI3Kpep treatment on promoting axon extension on CSPG substrata occur during the first 0–18 min of treatment, and the rate of axon extension ramps up during this period.

Treatment with PI3Kpep promotes growth cone protrusive dynamics on CSPG substrata

In order to determine the effects of PI3Kpep treatment on the dynamics of growth cones on CSPG substrata, we acquired phase contrast time-lapse movies of growth cones using 6 second inter-frame intervals. Imaging was commenced at 30 min post treatment with PI3Kpep or PI3KpepAla for a period of 5 min. Growth cones treated with PI3KpepAla exhibited behaviors consistent with those described by Tom et al. (2004), which are referred to as dystrophic. The growth cones were able to generate lamellipodial and filopodial protrusions, but these protrusions were highly unstable and soon retracted back into the growth cone (Figure 5A; $n=14$). In contrast, growth cones treated with PI3Kpep exhibited more protrusive activity and the lamellipodia persisted for significantly longer time periods (Figure 5B; $n=15$). Qualitatively, the effects of PI3Kpep became detectable at 15–20 min post-treatment. Analysis of growth cone dynamics at 30 min post-treatment revealed that growth cones exhibited lamellipodia for a total of 40 ± 13 (mean $\pm$ SEM; PI3KpepAla) and 160 ± 19 (PI3Kpep) seconds during the 300 sec imaging period ($p < 0.0001$). Analysis of the rate of filopodia formation at growth cones revealed that, on CSPGs, PI3KpepAla and PI3Kpep treated growth cones formed 3.2 ± 1.0 and 6.9 ± 1.0 filopodia/min, respectively (mean $\pm$ SEM; $p < 0.003$).

Expression of R-Ras promotes axon extension and restores growth cones on CSPG substrata

R-Ras in a non-oncogenic form of the Ras family of GTPases which is strongly expressed in neurons and is activated by NGF (Oinuma et al., 2004; Iwashita et al., 2007), and acts as an upstream activator of PI3K (Marte et al., 1997). Immunostaining of sensory neuron cultures with anti-R-Ras antibodies, to detect endogenous R-Ras, revealed signal present in the cell bodies, along distal axons and in growth cones (Figure 6A, B). The N-terminus of R-Ras is a determinant of PI3K-mediated Rac1 activation (Holly et al., 2005). We therefore determined the effects of expressing R-Ras and an N-terminus deletion (N-R-Ras) in sensory neurons cultured on laminin or laminin + CSPGs. For all experiments involving R-Ras we used the G38V constitutively active mutant form of R-Ras to drive its activity independent of activation by extracellular signals (Wurtzel et al., 2012). All R-Ras constructs were tagged

with GFP for detection of expression. Western analysis of pAkt (Ser473) levels in serum starved NIH3T3 cells expressing R-Ras and N-R-Ras confirmed that full length G38V R-Ras increased pAkt levels to greater levels than N-R-Ras (Figure 6C). R-Ras increased pAkt levels by over 12 fold over GFP control, in contrast to N-R-Ras which increased levels by approximately 0.6 fold. Expression of the N-terminus of R-Ras alone did not affect the levels of pAkt (Figure 6C), indicating that this domain is required but not sufficient for the elevation of pAkt levels induced by full length R-Ras.

We first determined the effects of expressing R-Ras on axon morphogenesis and growth cone morphologies using E7 sensory neurons. In order to determine the effects of R-Ras expression in the absence of NGF-induced signaling, experiments were performed using neurons cultured on a laminin substratum in the absence of NGF. Expression of R-Ras promoted a substantial increase in the percentage of growth cones that exhibited a spread morphology, characterized by lamellipodia and filopodia, while decreasing the percentage that were collapsed (3 filopodia) (Figure 6D). However, N-R-Ras did not promote the spreading of growth cones (Figure 6D). In contrast, N-R-Ras increased the percentage of growth cones exhibiting filopodia, but not lamellipodia, and decreased the percentage of collapsed growth cones (Figure 6D). These data suggest that PI3K activity downstream of R-Ras promotes growth cone spreading. However, the PI3K-activation deficient N-R-Ras can still promote the formation or retention of filopodia at growth cones. Expression of R-Ras also increased axon lengths by approximately 260% (Figure 6E), and expression of N-R-Ras increased axon lengths to a level comparable to that of R-Ras (Figure 6E). These observations indicate that R-Ras is a strong positive regulator of embryonic growth cone morphology and axon extension on laminin substrata in the absence of NGF signaling. Based on the similarity of the effects of full length R-Ras relative to N-R-Ras on the length of axons, the effects of R-Ras on axon extension are unlikely to be primarily due to activation of PI3K signaling.

To determine the effects of expressing R-Ras on axon extension on substrata coated with CSPGs, we used two concentrations of CSPGs determined to have maximal (100 $\mu\text{g/mL}$) and intermediate (50 $\mu\text{g/mL}$) effects on the extension of dissociated neurons (Figure 7A, B). Expression of R-Ras promoted both the percentage of neurons that gave rise to axons (Figure 7A) and the length of axons that formed (Figure 7B). R-Ras-expressing neurons on the intermediate dose of CSPGs (50 $\mu\text{g/mL}$) developed axons of similar lengths to neurons cultured in the absence of CSPGs, but in otherwise identical culturing conditions (Figure 7B). On substrata coated with CSPGs (50 and 100 $\mu\text{g/mL}$), expression of R-Ras maintained the percentage of axon tips exhibiting a spread morphology, characterized by lamellipodia and filopodia, to baseline levels (Figure 7C). Analysis of the number of filopodia present along the distal 20 μm of axons, reflective of the growth cone and 10–15 μm of distal axon, revealed that R-Ras expression restored filopodia on CSPG substrates to baseline levels (Figure 7D). Collectively, these observations demonstrate that expression of activated R-Ras greatly minimizes the effects of CSPGs on sensory axons and their growth cones.

In order to gain insights into the role of PI3K signaling downstream of R-Ras, we expressed the N-R-Ras construct and plated neurons on substrata coated with 50 $\mu\text{g/mL}$ CSPGs. Unlike expression of wild type R-Ras, expression of N-R-Ras failed to rescue the

percentage of spread growth cones on CSPGs (Figure 7C). Expression of N-R-Ras partially restored the number of filopodia along distal axons (Figure 7D). Similarly, relative to wild type R-Ras, expression of N-R-Ras resulted in a partial rescue of the percentages of neurons extending axons (Figure 7A). N-R-Ras increased axon lengths on 50 $\mu\text{g/mL}$ CSPGs to the same extent as wild type R-Ras (Figure 7B). Collectively, these data indicate that R-Ras activity promotes axon extension on CSPGs mostly independent of PI3K activation, but that R-Ras-driven PI3K activity is required to restore growth cone morphology on CSPGs.

DISCUSSION

CSPGs are important modulators of nervous system development, and they impair the regeneration and branching of axons following injury. The current study reveals that CSPGs impair PI3K signaling in embryonic sensory axons, and that aspects of the effects of CSPGs on axons can be overcome through the direct activation of the PI3K and R-Ras signaling pathways. These observations have implications for understanding the molecular basis of how CSPG signaling assists in the sculpting of the developing nervous system (Kwok et al, 2012), and suggest therapeutic targets for promoting axon regeneration or sprouting in the context of injury (García-Alías and Fawcett, 2012).

Recent work has identified receptors that mediate CSPG signaling. Two of these receptors are phosphatases (LAR and PTP σ), and two of the Nogo receptors also appear to mediate aspects of CSPG signaling (Coles et al., 2011; Fisher et al., 2011; Sharma et al., 2012). We have previously reported that both LAR and PTP σ are expressed by embryonic chicken sensory neurons and are present at the growth cone and along the axon (Ketschek et al, 2012). The current study demonstrates that CSPGs decrease PI3K signaling through Akt and S6 in axons. The mechanism used by CSPG receptors to attenuate signaling by the PI3K pathway remains unclear. Semaphorins can decrease net cellular levels of PI3K signaling by inactivating neurotrophin signaling through the PI3K pathway (Atwal et al., 2003). It remains to be determined if CSPG receptors may operate through a similar mechanism. Relatedly, R-Ras is potently inhibited by semaphorins through plexin-mediated R-RasGAP activity (Oinuma et al., 2004; Pasterkamp, 2005), and we found that R-Ras can bypass CSPGs in growth cone spreading and axon elongation; hence, R-Ras may be a common regulatory node in CSPG or semaphorin blockade of these processes. Our data reveal that pAkt levels are decreased in axons by CSPGs. PI3K phosphorylates the lipid Phosphatidylinositol 4,5-bisphosphate (PIP₂) to Phosphatidylinositol (3,4,5)-trisphosphate (PIP₃), which then recruits PDK1 and Akt to the membrane resulting in PDK1- and mTORC2-mediated phosphorylation of Akt (Matheny and Adamo, 2009). It is thus possible that CSPGs may be acting through inhibiting PI3K directly. Alternatively, CSPGs may inhibit PDK1 downstream of PI3K, or impair the function of upstream activators of PI3K. These two mechanisms could be addressed by determining if CSPGs affect PIP₃ levels in neurons, and thus likely act at the level of regulating the activity of PI3K or its antagonistic phosphatase PTEN (Crino, 2011), which converts PIP₃ to PIP₂. The observation that direct activation of PI3K can reverse the effects of CSPGs, including restoring pAkt levels, suggests that PDK1 may not be a target of CSPG signaling. Rather, the data indicate that CSPG signaling acts to prevent PI3K activity, blocking the pathway at its first step.

Semaphorin 3A has been shown to regulate PTEN targeting to the leading edge of growth cones thus suppressing net PI3K-dependent PIP₃ levels (Chadborn et al., 2006), but whether CSPGs may similarly regulate PTEN localization, or possibly activity through regulation of RhoA-RhoA Kinase signaling (Sharma et al., 2012), remains to be determined. Interestingly, one of the receptors for CSPGs, LAR, has been shown to positively regulate Akt signaling through TrkB neurotrophin receptors in the absence of CSPG signaling (Yang et al., 2006). It will be of interest to determine if CSPG binding to LAR may block the positive regulation of Trk receptor signaling by LAR.

PI3K signaling regulates the cytoskeleton during axon development and chemotaxis (Sasaki and Firtel, 2006; Cosker and Eickholt, 2007; Waite and Eickholt, 2010; Gallo, 2011). WAVE proteins are activators of the Arp2/3 actin nucleating complex, which contributes to the formation of lamellipodia and filopodia in neurons (Gallo, 2013), and WAVEs are activated by PI3K-Rac1 signaling (Mack and Eickholt, 2011). NGF induces the PI3K-dependent intra-axonal synthesis of WAVE in sensory axons, which in turn contributes to axon branching (Spillane et al., 2012). R-Ras is also a potent stimulator of Rac1 activation through activation of PI3K (Berrier et al., 2000; Goldfinger et al., 2006; Wozniak et al., 2005). Although not directly addressed, the PI3K-Rac1-WAVE-Arp2/3 pathway may contribute to the effects of PI3K^{pep} and R-Ras on growth cone morphology observed in this study. Consistent with this notion, the N-R-Ras construct, which is deficient in PI3K-mediated Rac1 activation (Holly et al., 2005), failed to promote growth cone spreading both on permissive substrata and CSPGs. Interestingly, the axonal translation of WAVE proteins in response to NGF requires PI3K signaling (Spillane et al., 2012). Thus, the PI3K signaling axis may be promoting both the axonal synthesis and activation of WAVE-Arp2/3 in axons and growth cones. It remains to be addressed if and how CSPGs regulate WAVE levels or activity.

We identify that the levels of pS6 were also decreased in axons by CSPGs. S6 is a ribosomal protein which, in a phosphorylation-dependent manner, regulates the translation of a subset of mRNAs, and recent studies indicate the presence of a set of specialized ribosomes which differentially translate mRNAs based on their protein composition (Xue and Barna, 2012). A phospho-proteomic screen of the effects of CSPGs on cerebellar granule neurons revealed that the phosphorylation state of multiple cytoskeletal proteins and proteins involved in mRNA binding and translation is affected by CSPGs (Yu et al., 2012). A recent study provided evidence that CSPGs induce the axonal translation of the RhoA GTPase (Walker et al., 2012), a GTPase which is generally considered to inhibit axon extension (Sharma et al., 2012). The observation that CSPGs decrease pS6 levels may thus, at face value, be considered inconsistent with the induction of RhoA translation. However, as noted above, S6 regulates translation of a set of mRNAs, but not all. Thus, while CSPGs may induce the translation of RhoA in axons, the observed decrease in pS6 levels suggests that the translation of other sets of axonal mRNAs may be impaired by CSPGs. We have recently reported that NGF induces the PI3K-mTOR-dependent axonal translation of mRNA coding for proteins with roles in promoting axon extension and branching (e.g., the cytoskeletal proteins Arp2, WAVE and cortactin; Spillane et al., 2012). Interestingly, PI3K^{pep} treatment strongly induced increases in WAVE protein levels in axons in the absence of NGF signaling (Spillane et al., 2012), similar to the observed higher than expected increase in S6

phosphorylation levels observed in the current study. It will be of interest to examine further if WAVE mRNA is a preferential target for S6 mediated translation in axons. Additional work will be required to determine if CSPGs suppress translation of mRNAs coding for these growth promoting proteins, while at the same time promote translation of mRNAs coding for growth inhibitory molecules. It will be of interest to determine if CSPGs may bi-directionally regulate the axonal proteome through the regulation of intra-axonal protein synthesis, and possibly degradation.

Ras GTPases activate multiple downstream effector pathways including PI3K, and among the Ras superfamily R-Ras is a particularly strong activator of PI3-kinases (Marte et al., 1997; Rodriguez-Viciana et al., 2004; Goldfinger, 2008; Wurtzel et al., 2012). Expression of constitutively active R-Ras countered the effects of CSPGs on axon extension and growth cone morphology. However, the N-R-Ras construct used in this study, which is deficient in activation of PI3K relative to full length R-Ras, promoted axon extension on CSPGs to levels similar to those of full length R-Ras, but it did not restore growth cone morphology. This observation suggests the dissociation of the effects of CSPGs on growth cone dynamics and morphology from their effects on extension of the axon. This is not completely unexpected as sensory axons can grow in the absence of actin filament-based growth cones (Marsh and Letourneau, 1984; Letourneau et al., 1987; Jones et al., 2006), but under these conditions they fail to undergo guidance in response to borders between CSPGs and permissive substrata (Challacombe et al., 1996). Thus, R-Ras expression may have also promoted axon extension on CSPGs through regulation of one or more additional pathways, such as through RLIP76, Ral or Rho signaling (Goldfinger et al., 2006; Yu and Feig, 2002; Jeong et al., 2005; Wozniak et al., 2005). Afadin, a Ras effector, was described recently as a downstream effector of R-Ras in axon branching (Iwasawa, et al., 2012). Activation of the Ras effector Raf can promote axon extension (Markus et al., 2002); however, R-Ras, even when activated as in the current study, is not a strong activator of Raf (Marte et al., 1997; Self et al., 2001; Goldfinger, 2008). Ras signaling may control pS6 levels in axons. S6 is an established target of Ras-induced ERK and Akt signaling (Carriere et al., 2008), suggesting that R-Ras signaling could promote S6 phosphorylation in axons and may thus bypass PI3K signaling to control this shared effector. It will be of interest to determine if Ras signaling may contribute to NGF-PI3K signaling-mediated intra-axonal protein synthesis (Spillane et al., 2012), and whether the latter is affected by CSPGs.

The current data indicate that R-Ras regulates growth cone morphology in a manner dependent on PI3K signaling, as revealed by differences in the effects of full length and N-R-Ras on both permissive and CSPG substrata. Interestingly, the PI3K-dependent effects of R-Ras on growth cones did not appear to be required for R-Ras to promote axon extension. However, PI3K_{pep} treatment promoted both growth cone dynamics and axon extension on CSPGs, in the presence of NGF. This observation suggests that PI3K_{pep} may be restoring PI3K signaling in the context of other NGF activated pathways and thus promoting axon extension. Additional analysis will be required to further dissect the roles of PI3K and additional pathways in the regulation of growth cone dynamics and axon extension on CSPGs. Finally, our study used a mixture of chicken brain CSPGs consisting of neurocan, phosphacan, versican, and aggrecan. Different variants of the same proteoglycan (e.g., aggrecan) can differentially affect growth cone behaviors, and it will be of interest to

determine which CSPG, and which variant(s) of the CSPG, is mediating the effects detailed in this study (Beller et al., 2013).

PTEN is the phosphatase that converts PIP3 to PIP2 and thus counters PI3K signaling. Inhibition of PTEN function promotes both axon sprouting and regeneration in the injured spinal cord (Park et al., 2008; Liu et al., 2010; Christie et al., 2010). Our studies were performed using embryonic neurons, and it remains to be determined if promotion of PI3K activity using PI3Kpep in adult neurons will similarly counter the inhibitory effects of CSPGs. Pharmacological inhibition of PI3K in cultured embryonic and adult dorsal root ganglion neurons, the latter having been subjected to an in vivo conditioning lesion prior to culture, impairs the extension of axons from embryonic but not adult neurons on permissive substrata (Liu and Snider, 2001). Thus, PI3K may have a different requirement in the extension of axons from adult neurons than embryonic neurons. In contrast, in vitro inhibition of PTEN promotes the extension of axons from adult sensory neurons both with and without a conditioning lesion on permissive substrata (Christie et al., 2010), suggesting that potentiating PI3K signaling in adult sensory may also have beneficial effects on axon extension on CSPGs. However, in the interpretation of studies involving manipulation of PTEN it is cautious to consider that PTEN also exhibits protein phosphatase activity toward focal adhesion kinase (Tamura et al., 1998, 1999), and thus any effect of PTEN inhibition may also be mediated by changes in focal adhesion kinase function. Future studies will be required to address these issues and determine if activation of PI3K in adult sensory neurons using PI3Kpep will promote axon extension on CSPGs in a manner similar to that observed for embryonic neurons in this study.

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FIGURE 1.

CSPGs decrease the levels of axonal pAkt and pS6. Representative examples of axons and growth cones stained with anti-pAkt (**A**) and anti-pS6 (**B**), and counterstained with rhodamine phalloidin to reveal actin filaments. Neurons were cultured in the absence of NGF and then treated with 40 ng/mL NGF or control vehicle for 60 min. NGF treatment elevates the level of staining in axons and growth cones. (**C**) Quantification of the levels of pAkt and pS6 staining levels in distal axons. For each antibody, data are shown for +/- NGF treatment (E7) and +/- CSPG substrata (E14) in the presence of NGF. n=49–68 axons were sampled per group from 3–4 independent cultures.

FIGURE 2.

PI3Kpep increases the levels of axonal pAkt and pS6 on CSPG substrata. E14 sensory explants were cultured in the presence of NGF on substrata +/- CSPGs are then treated with PI3Kpep or the control PI3KpepAla for 1 hr. The levels of pAkt and pS6 were quantified in axons as in Figure 1. n=42-49 axons were sampled per group from 3-4 independent cultures.

FIGURE 3.

PI3Kpep restores growth cone morphology on CSPG substrata and promotes axon crossing onto CSPG substrata. **(A)** Examples of E14 axons and growth cones stained with anti- α -tubulin and rhodamine phalloidin to reveal actin filaments. On CSPG substrata axons exhibited simplified growth cones with fewer filopodia and less spreading/lamellipodia formation. Treatment with PI3Kpep, relative to PI3KpepAla, for 1 hr restored growth cones on CSPGs to morphologies similar to those observed on control substrata. **(B)** Quantification of growth cone morphologies as a function of substratum and PI3Kpep and PI3KpepAla treatment. $n > 43$ axons per group, 3–4 cultures per group. Growth cone areas are shown in blue, and filopodia number in red, both as percent of control. **(C)** Example of axons encountering borders between permissive and CSPG substrata in the presence of PI3KpepAla or PI3Kpep. Axons were stained with anti- α -tubulin and CSPGs were stained as described in Ketschek et al (2012). In the presence of PI3KpepAla axons did not extend onto the CSPG-coated substratum, and instead straddled the border between the substrata. In contrast, in the presence of PI3Kpep axons often crossed onto the CSPG coated substratum. **(D)** Quantification of the ratio of axons crossing onto the CSPG-coated substratum relative to those that did not, and straddled the border. χ^2 analysis on proportion of axons crossing. $n = 6$ explants per group.

FIGURE 4.

PI3Kpep promotes axon extension on CSPG substrata. (A) 20x composites of axons extending from dorsal root ganglion explants on CSPG-coated substrata overnight in the continuous presence of PI3Kpep or PI3KpepAla. In the presence of PI3Kpep explants exhibited greater densities of axons and the axons extended longer distances than in the presence of PI3KpepAla. (B) Quantification of the number of axons crossing lines at 300 and 400 μm from the base of explants in the representative experiments depicted in panel (A). $n=8$ explants per group. (C) Examples of axons and growth cones, cultured in NGF and extending on a CSPG-coated substratum, from time-lapse sequences commenced upon treatment with PI3Kpep or PI3KpepAla. The white arrowheads in the 90 min panels denote the position of the growth cone (extent of phase dark axon shaft) at 0 min. (D) Measurement of the distance axons extended during a 90 min period in the experiments depicted in panel (C). PI3Kpep treated axons extended approximately 3 fold faster than those treated with PI3KpepAla. $n=29$ and 18 axons for PI3KpepAla and PI3Kpep groups from 3 and 4 cultures, respectively. (E) Measurement of the distance axon extended during the first 0–9 and 9–18 min following treatment with PI3KpepAla or PI3Kpep. Dunn multiple comparisons test.

FIGURE 5.

PI3Kpep treatment promotes growth cone dynamics on CSPG substrata. **(A)** Time-lapse stills (seconds denoted in panels) of a representative growth cone on a CSPG substratum treated with PI3KpepAla. Note that although lamellipodia formed (e.g., arrowheads at 54 and 162 sec) they were transient and readily retracted into the growth cone. **(B)** Example of a growth cone treated with PI3Kpep. The panels follow the same format as in (A). In comparison to (A), note the persistence of lamellipodia and that the growth cone exhibits lamellipodia throughout most of the sequence.

FIGURE 6.

Effects of R-Ras and N-R-Ras ectopic expression on the length and morphology of sensory neuron axons on laminin substrata in the absence of NGF. **(A)** Examples of the pattern of anti-R-Ras staining in cultured E7 sensory neurons. R-Ras (green) was evident in cell bodies and along axons. **(B)** Examples of R-Ras staining in distal axons. The R-Ras signal was punctate and present throughout the axon shaft, central and peripheral domains of growth cones. R-Ras was present in both lamellipodia and filopodia. **(C)** Western analysis of pAkt phosphorylation in serum starved NIH 3T3 cells expressing R-Ras constructs. As a positive control, a 30 treatment with bovine calf serum elevated pAkt levels in cells transfected with the empty vector (vector), relative to serum starved cells. Expression of full length R-Ras in the absence of serum treatment also increased pAkt levels. Expression of the N-terminus (N-term) of R-Ras did not appreciably affect pAkt levels. Expression of N-R-Ras slightly elevated pAkt levels relative to vehicle controls not treated with serum, but to a much lower extent than full length R-Ras. Blot is representative of 3 independent experiments. Quantitative analysis of pAkt/Akt levels, relative to serum starved control conditions, is shown in the bar graph adjacent to the blot. A value of 1 on the Y-axis is reflective of baseline control levels. **(D)** Expression of R-Ras and N-R-Ras affects growth cone morphology. R-Ras expression resulted in a greater percentage of growth cones with spread

lamellipodial morphologies, while N-R-Ras expression increased the percentage of growth cones exhibiting filopodia (p values for comparisons to GFP controls). The distribution of growth cone morphologies also differed between R-Ras and N-R-Ras ($p<0.001$). **(E)** Expression of R-Ras and N-R-Ras increased the length of sensory neuron axons. The lengths of axons expression R-Ras and N-R-Ras did not differ.

Figure 7.

Effects of R-Ras and N-R-Ras expression on the morphology of growth cones and the length of sensory neuron axons on CSPG substrata. A concentration of 5 ng/mL NGF was present in all experimental groups to provide neurotrophic support. **(A)** Distributions of neurons with no axons, axons < 30 μm and axons > 30 μm. Transfected neurons were sampled from six cultures per group. See Table I for statistical analysis. Data is presented as percentages in the graph. **(B)** Measurements of axon lengths. Gray lines in bars reflect the median. Numbers reflect the sample size for axon measurements. On 100 μg/mL CSPG very few neurons generated axons >30 μm, the inclusion criteria (n=4; see Panel A). See Table II for statistical analysis. **(C)** Percentages of axon tips exhibiting spread growth cones. See Table III for statistical analysis. **(D)** Number of filopodia along the distal 20 μm of axons. See Table IV for statistical analysis.

TABLE I

Statistical analysis of distributions of neuronal morphologies in Figure 7A.

<u>Comparison</u>	<u>p value</u>
GFP vs. GFP 50 µg/mL CSPG	<0.001
GFP vs. GFP 100 µg/mL CSPG	<0.001
GFP vs. R-Ras 50 µg/mL CSPG	>0.05
GFP vs. R-Ras 100 µg/mL CSPG	<0.001
GFP 50 µg/mL CSPG vs. R-Ras 50 µg/mL CSPG	p<0.0001
GFP 50 µg/mL CSPG vs. N-R-Ras 50 µg/mL CSPG	p<0.002
GFP 100 µg/mL CSPG vs. R-Ras 100 µg/mL CSPG	p<0001

χ^2 tests for independence used for comparisons using raw categorical cell counts.

TABLE II

Statistical analysis of axon lengths in Figure 7B.

<u>Comparison</u>	<u>p value</u>
GFP vs. GFP 50 µg/mL CSPG	<0.001
GFP vs. GFP 100 µg/mL CSPG	<0.01
GFP vs. R-Ras 50 µg/mL CSPG	>0.05
GFP vs. R-Ras 100 µg/mL CSPG	<0.05
GFP 50 µg/mL CSPG vs. R-Ras 50 µg/mL CSPG	<0.001
GFP 50 µg/mL CSPG vs. N-R-Ras 50 µg/mL CSPG	<0.01
GFP 100 µg/mL CSPG vs. R-Ras 100 µg/mL CSPG	>0.05

Kruskal-Wallis Test with Dunn's post-hoc tests.

TABLE III

Statistical analysis of growth cone spreading in Figure 7C.

<u>Comparison</u>	<u>p value</u>
GFP vs. GFP 50 µg/mL CSPG	<0.05
GFP vs. GFP 100 µg/mL CSPG	<0.001
GFP vs. R-Ras 50 µg/mL CSPG	>0.2
GFP vs. R-Ras 100 µg/mL CSPG	>0.5
GFP 50 µg/mL CSPG vs. R-Ras 50 µg/mL CSPG	<0.05
GFP 50 µg/mL CSPG vs. N-R-Ras 50 µg/mL CSPG	>0.5
GFP 100 µg/mL CSPG vs. R-Ras 100 µg/mL CSPG	>0.01

χ^2 tests for independence used for comparisons using raw categorical cell counts.

TABLE IV

Statistical analysis of number of filopodia in Figure 7D.

<u>Comparison</u>	<u>p value</u>
GFP vs. GFP 50 µg/mL CSPG	<0.001
GFP vs. GFP 100 µg/mL CSPG	<0.05
GFP vs. R-Ras 50 µg/mL CSPG	>0.05
GFP vs. R-Ras 100 µg/mL CSPG	>0.05
GFP 50 µg/mL CSPG vs. R-Ras 50 µg/mL CSPG	<0.001
GFP 50 µg/mL CSPG vs. N-R-Ras 50 µg/mL CSPG	>0.05
GFP 100 µg/mL CSPG vs. R-Ras 100 µg/mL CSPG	<0.01

Kruskal-Wallis Test with Dunn's post-hoc tests.