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Upregulation of reggie-1/flotillin-2 promotes axon regeneration in the rat optic nerve in vivo and neurite growth in vitro

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A B S T R A C T

The ability of fish retinal ganglion cells (RGCs) to regenerate their axons was shown to require the re expression and function of the two proteins reggie 1 and 2. RGCs in mammals fail to upregulate reggie expression and to regenerate axons after lesion suggesting the possibility that induced upregulation might promote regeneration. In the present study, RGCs in adult rats were induced to express reggie 1 by intravitreal injection of adeno associated viral vectors (AAV2/1) expressing reggie 1 (AAV.R1 EGFP) 14d prior to optic nerve crush. Four weeks later, GAP 43 positive regenerating axons had crossed the lesion and grown into the nerve at significantly higher numbers and length (up to 5 mm) than the control trans duced with AAV.EGFP. Consistently, after transduction with AAV.R1 EGFP as opposed to AAV.EGFP, primary RGCs in vitro grew long axons on chondroitin sulfate proteoglycan (CSPG) and Nogo A, both glial cell derived inhibitors of neurite growth, suggesting that reggie 1 can provide neurons with the ability to override inhibitors of neurite growth. This reggie 1 mediated enhancement of growth was reproduced in mouse hippocampal and N2a neurons which generated axons 40 60% longer than their control counterparts. This correlates with the reggie 1 dependent activation of Src and PI3 kinase (PI3K), of the Rho family GTPase Rac1 and downstream effectors such as cofilin. This increased growth also depends on TC10, the GTPase involved in cargo delivery to the growth cone. Thus, the upregulation of reggie 1 in mammalian neurons pro vides nerve cells with neuron intrinsic properties required for axon growth and successful regeneration in the adult mammalian CNS.

Introduction

Neurons in the mammalian central nervous system (CNS) possess a very limited ability to regenerate axons after a lesion. This is in con trast to lower vertebrates in which injured CNS nerve fiber tracts are restored and functional connections re established. The failure of mammalian neurons to regenerate neurites is caused by the inhibito ry influence of glial cells in the environment of the lesioned axons and by the unfavorable neuron intrinsic properties (Liu et al., 2011). In fish, both of these extrinsic and intrinsic conditions are apparently optimized for axon regeneration (Stuermer, 2012). When retinal gan glion cells (RGCs) regenerate their axons in the fish visual system, they upregulate the expression of many proteins, including reggie 1 and reggie 2 (Schulte et al., 1997) which proved to be essential for axon growth in fish and mammalian neurons (Munderloh et al., 2009). Reggies, also known as flotillins (Bickel et al., 1997), are so called lipid raft/microdomain proteins which are associated with the cytoplasmic face of the plasma membrane and with abundant in tracellular transport vesicles (Stuermer, 2010). Reggies were implied in an exocyst and TC10 dependent targeted delivery of specific cargo to defined sites of the cell including the growth cone (Stuermer, 2010) and are involved in growth cone elongation (Munderloh et al., 2009). Recent results showed, accordingly, that reggies promote the recruitment of N cadherin to the growth cone in mammalian hip pocampal neurons in association with the cellular prion protein (PrP) (Bodrikov et al., 2011).

Interestingly, in older experiments reggie 1 and 2 were detected in the adult rat visual system specifically in those RGCs which succeed in regenerating their axons when the optic nerve is replaced by a periph eral nerve graft (Lang et al., 1998; Richardson et al., 1980). These obser vations suggest that upregulation of reggie in adult rats might improve the regenerative capabilities of CNS neurons. We have previously demonstrated that rat RGCs can be transduced by intravitreal injection of adeno associated viral vectors (AAV) and that virus mediated upregulation of pro regenerative proteins can improve the regenerative

Abbreviations: AAV, adeno-associated viral vector; CSPG, chondroitin sulfate proteoglycans; EGFP, enhanced green fluorescent protein; ONC, optic nerve crush; RGC, retinal ganglion cell.

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response of RGCs after optic nerve crush (ONC) lesion (Planchamp et al., 2008). Here, we evaluate if AAV mediated upregulation of reggie 1 promotes regeneration of injured rat RGCs in vivo, despite the growth inhibitory environment in the adult optic nerve.

Our results demonstrate that reggie 1 upregulation in RGCs indeed promotes axon regeneration in the rat optic nerve following ONC in vivo and increases neurite length on the non permissive substrates CSPG and Nogo A in vitro. We show further that reggie 1 overexpression in mouse hippocampal neurons and N2a cells augments axon growth and does so by an increased activation of Src and PI3K, the Rho type GTPases Rac1 and TC10 and downstream actin regulators.

Materials and methods

Reagents, antibodies and plasmids

Antibodies (Abs) and their distributors were as follows: monoclonal (m) Abs against ESA/reggie 1 (BD Biosciences), GAP 43 (Abcam), GFP (Roche), polyclonal (p) Abs against alpha tubulin (Abcam), phospho Src Y416, phospho ERK1/2 Thr202/204, phospho p38 Thr180/Tyr182, phospho PI3K p85(Tyr458)/p55(Tyr199), phospho cofilin Ser3 (Cell Signaling), phospho cortactin Y466 (Millipore), secondary Abs for Western Blot analyses (Jackson ImmunoResearch) and for immunohistochemistry (Cy3, Dianova). The following inhibitors were used: PI3K inhibitor LY294002 (Cell Signaling), Rac1 and ROCK (Y 27632) inhibitors (Calbiochem) and Src kinase inhibitor PP2 (Sigma Aldrich). The reggie 1 EGFP (R1 EGFP) plasmid was described previously (Solis et al., 2007) and the pEGFP N1 (EGFP) vector (Clontech) served as control. A dominant negative (DN) construct for TC10 (T31N) was kindly provided by Alan Saltiel (University of Michigan, MI, USA). The ECFP TC10 DN constructs were generated by cutting the TC10 cDNA with BamHI and EcoRI from the original vector and cloning in frame into the BglII and EcoRI sites of the pECFP C1 plasmid (Clontech).

Cloning and production of adeno associated viral vectors

The pAAV 9(5)hSyn reggie 1 EGFP vector was generated by inserting in frame the rat reggie 1 cDNA into the AgeI restriction site upstream of the EGFP sequence within the control pAAV 9(5)hSyn EGFP CytbAS ohneNot vector (GenBank ID: HQ416702). Reggie 1 cDNA was amplified from the R1 EGFP plasmid by PCR using the primers R1 AgeI for 5′ caccggtatgggcaattgccacacggtg 3′ and R1 AgeI Rev 5′ caccggtacctgcgcaccagtggcattc 3′. The resulting vector was sequenced to confirm the identity of the reggie 1 EGFP fusion construct. The length of the insert between the ITRs was reduced with an EcoNI and XbaI digestion, blunting with Pfu polymerase and religation.

Production of AAV (hybrid serotype 2/1) was performed as described before (Zolotukhin et al., 1999). In brief, 293 HEK cells (Stratagene) were transfected with calcium phosphate, HEPES buffered saline and a 0.5:0.5:1:1 molar ratio of pAAV RC, pH21, pHELPER and the respective pAAV expression vector (pAAV 9(5)hSyn Reggie 1 EGFP or pAAV 9(5)hSyn EGFP CytbAS ohne Not (GenBank ID: HQ416702)). pAAV RC, pHELPER and the pAAV MCS cloning vector were obtained from Stratagene; the pH21 (pAAV1) expressing AAV serotype 1 capsids were a gift from Helen Fitzsimons (Neurologix, Inc. OSU Comprehensive Cancer Center, Columbus, OH) and Matthew During (Molecular Virology, Immunology, and Medical Genetics, Columbus, OH). Cells were harvested ~48 h after transfection and purified by dialysis and virus gradient centrifugation in iodixanol. Fast protein liquid chromatography (FPLC) was performed to obtain high titer viral stocks. Viral titers were determined by quantitative PCR. For each purified virus stock, transduction efficiency was analyzed in primary cultures of rat cortical neurons.

Primary rat RGC culture

RGCs were prepared from Wistar rat pups on postnatal d 7 and enriched by a two step panning protocol for Thy 1 to 99.5% purity (Barres et al., 1988). Four thousands cells in 500 µl RGC medium per well were plated in 24 well plates. Coverslips (Sarstedt) were coated with poly D lysine (Sigma Aldrich) and either laminin (20 µg/ml; Sigma Aldrich) or CSPG (50 µg/ml, Chemicon; major components: neurocan, aggrecan, phosphacan and versican), or laminin and the GST Nogo A delta 20 peptide (and GST as control), as described previously (Abdesselem et al., 2009). RGC medium was composed of serum free neurobasal medium (Gibco) supplemented with sodium pyruvate (Sigma Aldrich), glutamine, N acetyl cysteine, triiodothyronine, Sato (BSA, transferrin, progesterone, putrescine, sodium selenite; Gibco), forskolin (final concentration 10 mM), human BDNF (final concentration 50 ng/ml; Tebu), insulin (final concentration 5 mg/ml; Sigma Aldrich), CNTF (final concentration 10 ng/ml; Tebu) and B27 supplement.

Four hours after seeding, the medium in each well was reduced to 250 µl and AAV was added (0.5×10^8 transforming units (TU) per well). Two hundred and fifty microliters of fresh medium were added to each well after 24 h. Transduction efficacy of RGCs on DIV 3 was usually >90%. On DIV 5 phase contrast photos of 6 randomly chosen visual fields per well were taken at an inverted microscope (Axiovert, Zeiss). Axon lengths of RGCs from 3 independent experiments were quantified with the neurite tracing module of the ImageJ plugin NeuronJ (Meijering et al., 2004) and the results statistically evaluated (one way ANOVA followed by Dunnett's post hoc test with significance at $p<0.05$ and standard error of mean (SEM)).

Intravitreal virus injection, optic nerve crush, perfusion and tissue processing

Animals were treated according to the regulations of the local animal research council and legislation of the State of Lower Saxony, Germany. Adult female Wistar rats (200 300 g, Charles River) were anesthetized by an intraperitoneal injection of 10% ketamine (95 mg/kg body weight) and 2% xylazine (7 mg/kg body weight). Five microliters of AAV (12×10^8 TU) were injected intravitreally with a Hamilton syringe. Two weeks later, an optic nerve crush (ONC) was performed as described previously (Koch et al., 2011b).

Twenty eight days after ONC the animals were sacrificed, the optic nerve and eye bulb were removed en bloc after transcardial perfusion (250 ml PBS followed by 200 ml 4% PFA in PBS (pH 7.4)) and post fixed in 4% PFA for 1 h. The retina was dissected, flat mounted in 30% glycerol and examined for viral transduction efficacy (Supplementary Fig. 1). Longitudinal cryosections of the optic nerve (16 µm) were stained with GAP 43 (1:250, 4 °C overnight) and secondary Cy3 labeled Ab (45 min, 20 °C), counter stained with DAPI (4,6 diamidino 2 phenylindole; Sigma Aldrich), and mounted in Moviol (Hoechst).

Image acquisition and evaluation

Optic nerve sections were photographed with an Axioplan microscope (Zeiss) equipped with AxioVision Software (Zeiss) and the number of GAP 43 positive axons was counted at defined distances from the crush site. In total, 15 sections from 10 optic nerves in the AAV.EGFP group and 15 sections from 7 optic nerves in the AAV.R1 EGFP group were evaluated. Statistical analysis was done using one way ANOVA to compare both groups in total followed by a student's *t* test with standard error of mean (SEM) to compare both groups at the respective distances from the crush site.

Culture of primary mouse hippocampal neurons

Cultures of hippocampal neurons were prepared from 1 to 3 day old mice as previously described (Munderloh et al., 2009). Briefly,

hippocampi were isolated, digested and homogenized. Cells were plated on poly L lysine coated coverslips in Neurobasal A medium containing B27 supplement (Invitrogen). After 3 h, the medium was changed to Neurobasal A supplemented with B27, 0.5 mM L glutamine, 1% penicillin streptomycin and 5 ng/ml fibroblast growth factor (Invitrogen). Neurons were transfected with R1 EGFP or EGFP, or co transfected with ECFP TC10 DN using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were fixed in 4% PFA and mounted for microscopic analysis 48 h after transfection. Alternatively, 50 μM LY294002, 100 μM Rac1 inhibitor, 50 μmol Y 27632 or 100 μM PP2 were applied to live neurons 24 h after transfection. Neurons were cultured for additional 24 h, fixed and mounted. Wide field images of more than 100 transfected cells per group from three independent experiments were taken at an Axiovert 200 M (Zeiss) using a 40× Plan NEOFLUAR objective. Length of neurites was measured using the ImageJ software and subjected to statistical analysis (paired student's *t* test; mean ± SEM). In a third experiment, transfected hippocampal neurons were immunostained with Abs against phosphorylated cofilin, Src and PI3K. The staining intensity over growth cones was evaluated using ImageJ software.

N2a cell culture

Neuroblastoma N2a cells were cultured and transfected as described previously (Langhorst et al., 2008). Briefly, N2a cells were transfected with the R1 EGFP or EGFP for 24 h, cultured for additional 24 h on laminin coated coverslips in MEM containing 50 ng/ml insulin like growth factor (IGF 1) (Biomol), fixed in 4% PFA, and subjected to microscopic analysis. Wide field images of roughly 200 transfected cells per group from three independent experiments were analyzed as described above. Alternatively, transfected N2a cells were immunostained with Abs against phosphorylated PI3K, and the immunofluorescence intensity of the neurites was quantified using ImageJ software.

Western blot analyses

N2a cells were mock transfected or transfected with R1 EGFP or EGFP vectors for 48 h. Cells were then lysed in ice cold kinase lysis buffer (20 mM Tris HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, 1% (v/v) Triton X 100, 10% (v/v) glycerol) supplemented with a protease and phosphatase inhibitor cocktail (Thermo Scientific). Cleared lysates were directly used for Western blotting, further analyzed using ImageJ software and subjected to statistical analyses from 4 independent experiments (paired student's *t* test; mean ± SEM).

Results

Production of reggie 1 expressing AAV

To overexpress reggie 1 in neurons in vitro and in vivo, we produced AAV expressing a reggie 1/EGFP fusion construct (R1 EGFP) (Solis et al., 2007) under the control of the human synapsin promoter (AAV.R1 EGFP). A previously described AAV of the same type expressing EGFP only (Koch et al., 2011a) served as control (AAV.EGFP). A hybrid serotype (AAV2/1) consisting of the AAV2 inverted terminal repeats (ITR) packed into AAV1/AAV2 hybrid capsids (molar ration 1:1) was generated as this type results in a higher gene expression level in neurons than the traditional AAV2/2 (Blits et al., 2010; Dinculescu et al., 2005; Heilbronn and Weger, 2010; Lebherz et al., 2008) and leads to a more efficient and rapid transduction of RGCs in vitro and in vivo (own unpublished data). Expression of the transgene was restricted to neuronal cells by the use of the neuron specific synapsin promoter (Kugler et al., 2003).

Reggie 1 promotes RGC axon regeneration after optic nerve crush

To analyze whether upregulation of reggie 1 promotes RGC axon regeneration in vivo, we evaluated its effects in the rat optic nerve crush (ONC) model. The viral vectors were injected intravitreally 2 weeks before ONC to ensure sufficient expression of the transcript. Western blot analyses from extracts of transduced rat retinas and optic nerves showed that the R1 EGFP protein as well as endogenous reggie 1 is expressed in both tissues (Fig. 1A). Retinal whole mounts (see Supplementary Fig. 1 for an overview of a transduced retina) and cryosections of transduced retinas verified that R1 EGFP was specifically expressed in RGCs. The R1 EGFP fluorescence was weak in RGCs but amplification of the signal by anti EGFP immunostaining

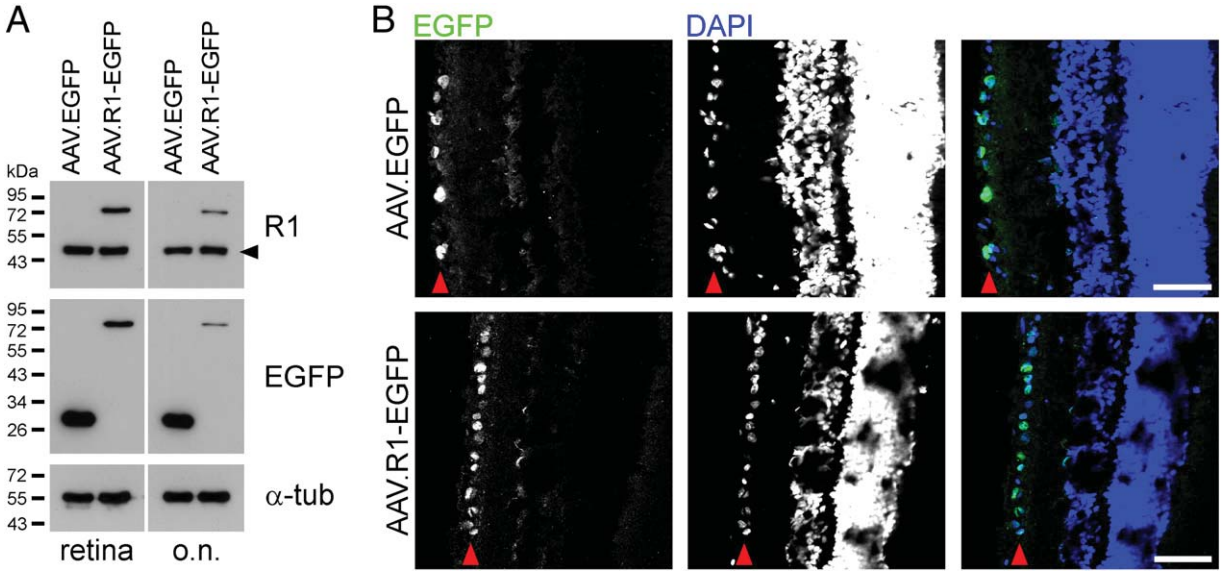

Fig. 1. AAV.R1-EGFP transduction of rat RGCs in vivo. (A) Western blot analysis with Abs against reggie-1 (R1) and EGFP on protein lysates of AAV.R1-EGFP and AAV.EGFP transduced retinas and optic nerves (o.n.) shows that both constructs were efficiently expressed. The black arrowhead indicates the presence of endogenous R1. An antibody against alpha-tubulin (a-tub) served as loading control. (B) Anti-EGFP immunostained cryosections from AAV.R1-EGFP and AAV.EGFP transduced retinas revealed the expression of both constructs in RGCs (red arrowheads). DAPI staining was used to display nuclei in retina layers. Scale bars, 50 mm.

showed that the expression of both R1 EGFP and control EGFP was specifically localized to RGCs (Fig. 1B).

To determine whether R1 EGFP expression is beneficial for axonal regeneration in vivo, a crush lesion of the optic nerve was performed in the intravitreally injected rats (Koch et al., 2011b). Four weeks after ONC, the optic nerves were stained with an antibody against GAP 43 to quantify the number of regenerating RGC axons (Schaden et al., 1994) at increasing distances from the crush site. Notably, transduction of retinas with AAV.R1 EGFP significantly increased the number of GAP 43 positive axons compared to AAV.EGFP at all examined distances distal to the crush site (Fig. 2). In the AAV.R1 EGFP treated animals, 3 5 times as many GAP 43 positive axons as in the controls had crossed the lesion site and had grown into the distal part of the optic nerve. Beyond 1000 µm distal from the lesion site, GAP 43 positive regenerating axons were rarely found in the control but did occur frequently in the AAV.R1 EGFP injected rats (number of optic nerve sections with GAP 43 positive axons >1000 µm distal from the lesion: AAV.EGFP: 2 of 15, AAV.R1 EGFP: 13 of 15). Moreover, in the AAV.R1 EGFP treated animals, single GAP 43 positive axons could often be followed up to 5 mm distal from the crush site (Fig. 2C) (number of optic nerve sec tions with GAP 43 positive axons >5000 µm distal from the lesion: AAV.EGFP: 0 of 15, AAV.R1 EGFP: 5 of 15).

These data show that upregulation of reggie 1 in rat RGCs signifi cantly promotes RGC axon regeneration into the rat optic nerve

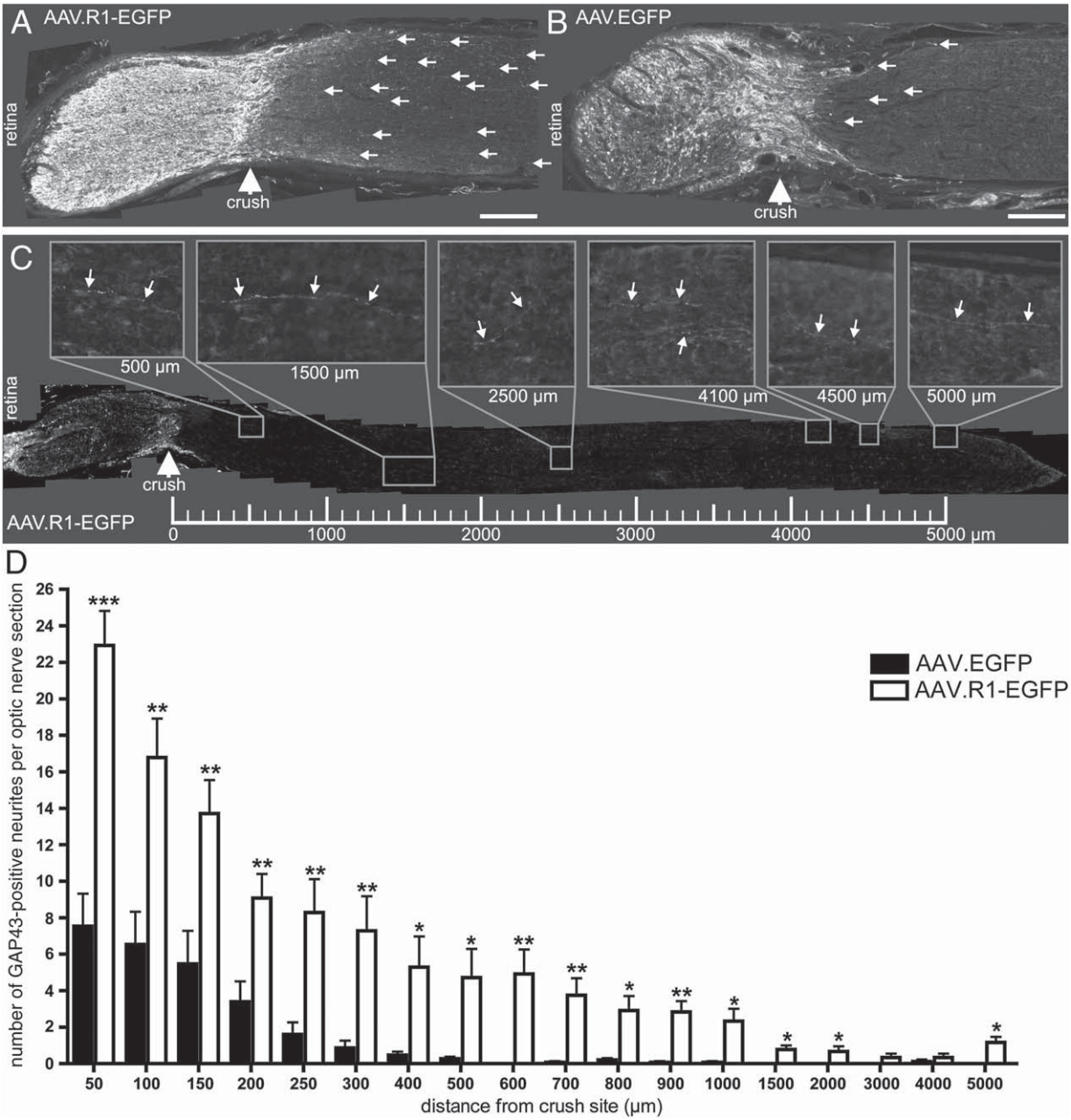

Fig. 2. AAV.R1-EGFP promotes axonal regeneration after ONC. (A,B) Composite pictures of the proximal part of the optic nerve 4 weeks after crush lesion (large white arrowhead) immunostained against GAP-43. After transduction with AAV.R1-EGFP (A) a substantial number of GAP-43 positive axons (white arrows) has grown over the crush site. In contrast, only few GAP43-positive neurites crossed the lesion site in the AAV.EGFP-control (B). (C) Overview of the proximal 6000 µm of a GAP-43 stained optic nerve 4 weeks after ONC and previously transduced with AAV.R1-EGFP (composite picture). Single GAP-43 positive axons can be followed over a distance of 5000 µm distal from the crush site depicted at higher magnifications in the frames above the nerve. D) Quantification of the number of GAP-43 positive axons at different distances from the crush site. Transduction with AAV.R1-EGFP significantly increased the number of axons at all analyzed positions along the nerve. One-way ANOVA followed by student's *t*-test for each point; *: $p<0.05$; **: $p<0.005$; ***: $p<0.0005$. Error bars, SEM.

(Fig. 2D) without the need of any additional treatment. This implies that increased reggie 1 expression improves the neuron intrinsic capability of rat RGCs to override the growth inhibitory environment in vivo and empowers adult mammalian neurons to re extend long axons.

Overexpression of reggie 1 enhances RGC axon growth on inhibitory substrates

One of the major factors that account for the lack of neuronal regeneration in the adult mammalian CNS is the inhibitory environment produced by the surrounding glia cells and the scar tissue. Molecular mediators of this repulsive action are, amongst others, chondroitin sulfate proteoglycans (CSPG) and Nogo A. To test whether upregulation of reggie 1 permits axon growth despite the presence of glial growth inhibiting molecules, we cultured primary RGCs of postnatal rats on coverslips coated with CSPG (major components: neurocan, aggrecan, phosphacan and versican) and Nogo A delta20, respectively. In both experiments the mean neurite length was determined in three independent experiments on day in vitro (DIV) 5 in 15 randomly chosen visual fields per condition (Fig. 3A). RGCs transduced with the control vector AAV.EGFP had a mean neurite length of 1409 μm (±64) on laminin and showed a 70% reduction to 430 μm (±32) when cultured on CSPG coated coverslips. RGCs transduced with AAV.R1 EGFP did not significantly increase axon length on laminin compared to AAV.EGFP (mean neurite length 1286±84 μm). On CSPG, however, RGCs transduced with AAV.R1 EGFP showed a significant, almost 1.8 fold increase in neurite length (771±39 μm) compared to the AAV.EGFP group (Fig. 3B) implying that upregulation of R1 EGFP improves the cell intrinsic conditions and renders RGCs capable of overcoming inhibitors.

Whether this would also apply to Nogo A was examined next by coating coverslips with laminin and GST fusion protein comprising the central delta20 region of the Nogo A protein (GST delta20) (Abdesselem et al., 2009), which was shown to inhibit axon outgrowth (Oertle et al., 2003). GST alone served as control (GST control). In RGCs transduced with AAV.EGFP, GST delta20 inhibited neurite outgrowth by 40% compared to the GST control (Fig. 3C). Transduction of the RGCs with AAV.R1 EGFP, however, completely counteracted the inhibitory effect of Nogo A delta20.

These results show that upregulation of reggie 1 enables postnatal RGCs to partially overcome CSPG and Nogo A delta20 mediated growth inhibition in vitro, consistent with the in vivo findings in the non permissive optic nerve.

Overexpression of reggie 1 in mouse hippocampal neurons and N2a cells results in increased neurite growth

Since reggie 1 upregulation exerted a stimulatory effect on neurite growth of RGCs in vivo and in vitro, we next analyzed whether reggie 1 expression also promotes neurite growth in other mammalian CNS neurons, namely primary mouse hippocampal neurons and N2a cells. The greater abundance of these neurons in cell culture allows for the analysis of downstream signaling molecules and for biochemical tests. Overexpression of R1 EGFP in hippocampal neurons resulted in a roughly 60% increase in the mean neurite length compared to control (mean neurite length: EGFP: 17.4±1.1 μm, R1 EGFP: 28.8±2.4 μm; $n=100$ cells per group) (Fig. 4). The number of neurites per cell was not affected meaning that R1 EGFP overexpression selectively promotes neurite elongation (mean neurite number per cell: EGFP: 2.7±0.05 μm, R1 EGFP: 2.6±0.06 μm). By contrast, in N2a cells R1 EGFP overexpression led to an increase in the number of neurites per cell

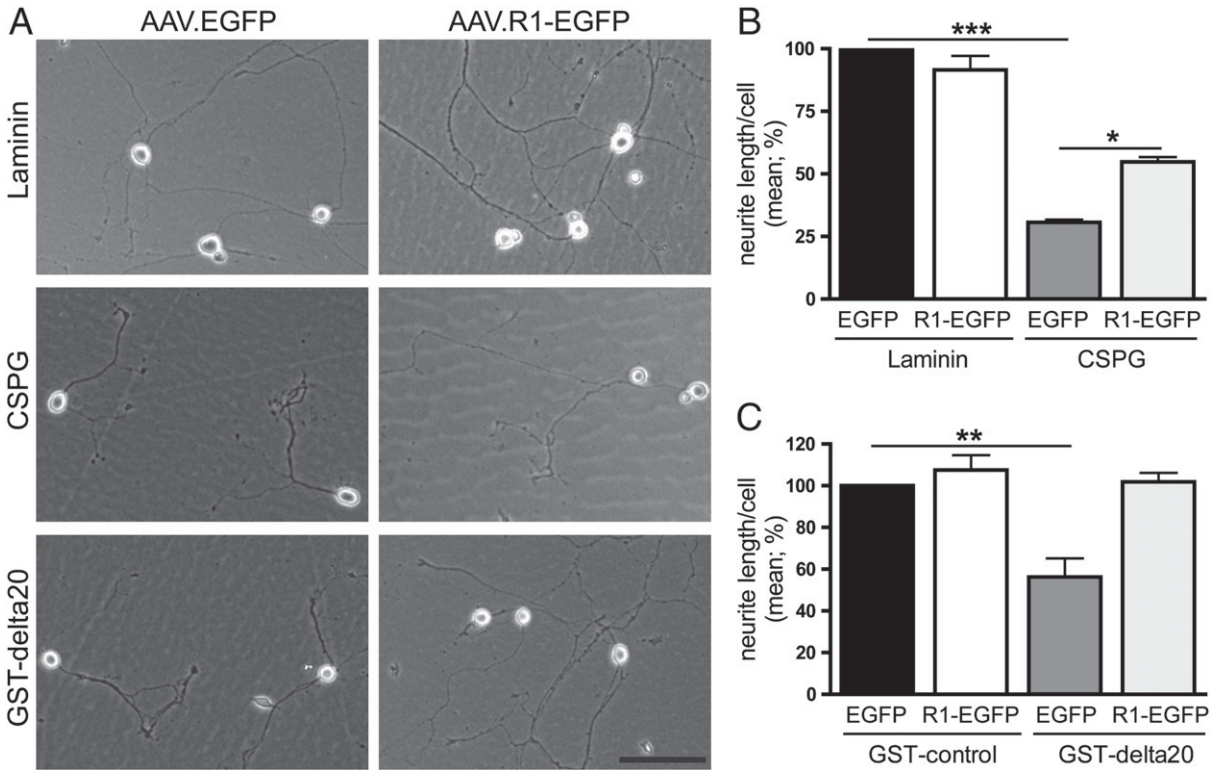

Fig. 3. Reggie-1 upregulation enhances neurite outgrowth of RGC on inhibitory substrates. (A) Phase contrast images of RGCs grown on laminin (upper row), the inhibitory substrate CSPG (middle row) or Nogo-A delta20 (lower row). RGCs transduced with AAV.EGFP (left column) show little neurite outgrowth on CSPG and Nogo-A delta20, whereas transduction with AAV.R1-EGFP (right column) leads to growth of long axons also on CSPG and Nogo-A delta20. Scale bar, 100 μm. (B,C) Quantification of the mean neurite length of RGCs on laminin or CSPG (B) and GST-control peptide or GST-delta20 (the inhibitory region of Nogo-A) (C) and transduced with AAV.EGFP or AAV.R1-EGFP on DIV 5. One-way ANOVA and Dunnett's post hoc test; ***: $p<0.0005$; **: $p<0.005$; *: $p<0.05$. Error bars, SEM.

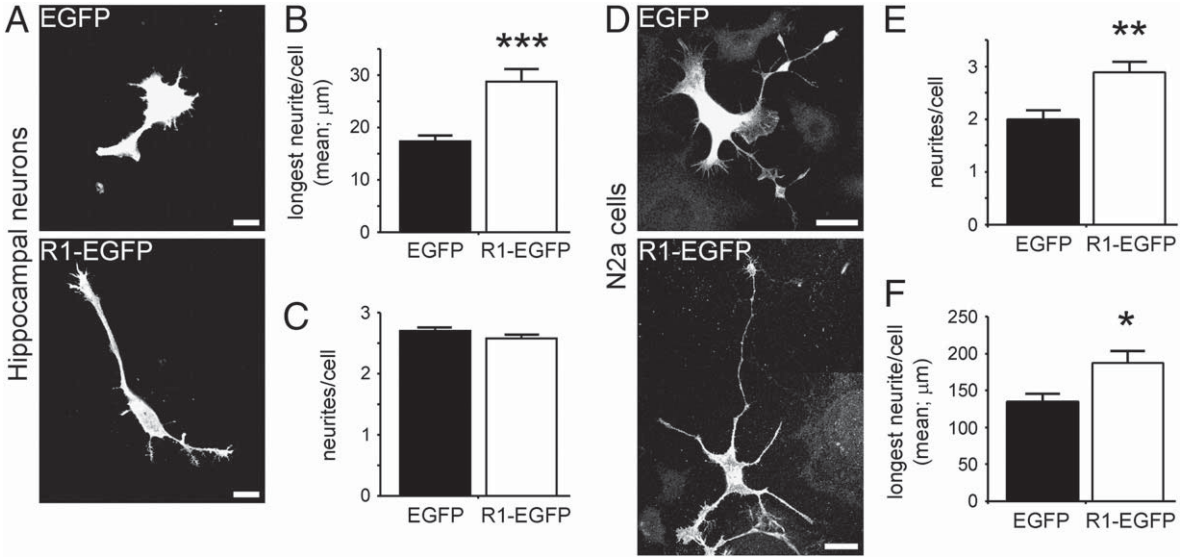

Fig. 4. Reggie-1 upregulation leads to increased neurite length in hippocampal neurons and N2a cells and increased number of neurites in N2a cells. (A) Mouse hippocampal neurons overexpressing reggie-1-EGFP (R1-EGFP) generated neurites twice as long as compared to neurons expressing EGFP control. (B,C) Quantification of axon length (B) and number of neurites per cell (C) of mouse hippocampal neurons transfected with the respective plasmids. (D) Representative images of N2a cells transfected with EGFP or R1-EGFP after 24 h stimulation with IGF-1. R1-EGFP overexpression (lower panel) induced a significant increase in the formation and length of neurites as compared to control EGFP cells (upper panel). (E,F) Quantification of number of neurites (E) and length of the longest neurite (F) in N2a cells overexpressing R1-EGFP or control EGFP. Student's *t*-test; ***: $p<0.001$, **: $p<0.01$; *: $p<0.05$. Error bars, SEM. Scale bars, 20 mm.

(mean neurite number per cell: EGFP: 2.0 ± 0.2, R1 EGFP: 2.9 ± 0.2) as well as to an approximately 40% increase in the mean length of the lon gest neurite compared to EGFP controls (mean neurite length: EGFP: 135 ± 11 µm, R1 EGFP: 187 ± 16 µm; n = 200 cells per group) (Figs. 4D, E, and F). Thus, reggie 1 overexpression not only enhances ax onal regeneration in lesioned rat RGCs in vivo but also augments neurite growth in mammalian CNS and N2a neurons in vitro.

Reggie 1 mediated increase in neurite length depends on the function of Src tyrosine kinase, ROCK, PI3K, Rac1 and TC10

According to our earlier results, downregulation of reggie 1 in neurons modulates the activation of the Rho family GTPases, their downstream effectors of actin cytoskeleton dynamics and Src tyrosine kinases (Bodrikov et al., 2011; Munderloh et al., 2009). Therefore, we examined whether overexpression of reggie 1 affects signaling in mammalian neurons. Hippocampal neurons were transfected with R1 EGFP or EGFP (control) and treated with inhibi tors specific for the Src tyrosine kinase, the Rho GTPase Rac1, PI3K, which is an upstream activator of Rac1 (Cosker and Eickholt, 2007), and ROCK. The first three inhibitors completely blocked the growth promoting effects of R1 EGFP on neurite length (Fig. 5). ROCK inhibi tion, however, increased the reggie 1 mediated increase in neurite length which is consistent with the notion that Rho dependent acti vation of ROCK inhibits growth (Fournier et al., 2003; Lingor et al., 2008; Niederost et al., 2002).

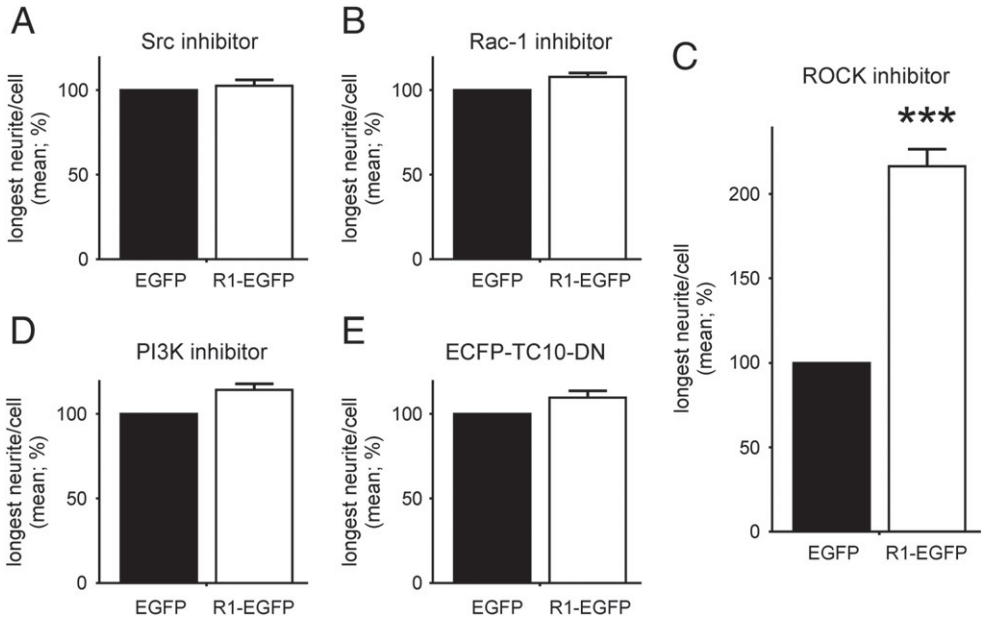

Fig. 5. Reggie-1 mediated increase in neurite length depends on the function of Src-tyrosine kinase, Rac1, ROCK, PI3K and TC10. Reggie-1-EGFP-transfected hippocampal neurons were exposed to inhibitors of Src tyrosine kinase (A), Rac-1 (B), ROCK (C), PI3K (D) which affects Rac-1, and the DN form of TC10 (E). The Src, Rac, PI3K inhibitors and DN TC10, but not the ROCK inhibitor, blocked the reggie-1-mediated gain in axon length (Fig. 4). Student's *t*-test, ***: $p<0.001$, Error bars, SEM.

It has recently been postulated that reggies affect neuronal differ entiation and regeneration by regulating the targeted delivery of bulk membranes and specific proteins from intracellular compartments to specific sites of the plasma membrane in a process involving the small GTPase TC10 (Stuermer, 2010). Thus, we tested whether a dominant negative (DN) mutant of TC10 (ECFP TC10 DN) would block the increase in neurite length induced by reggie 1 overexpression by co transfecting hippocampal neurons with ECFP TC10 DN and either EGFP (control) or R1 EGFP. Indeed, the increase in neurite length caused by reggie 1 overexpression was completely inhibited by the TC10 DN mutant (Fig. 5E).

Taken together, these data demonstrate that the growth promot ing effect of reggie 1 on neurites crucially depends on the function of Src tyrosine kinases, PI3K and the small GTPases Rac1 and TC10.

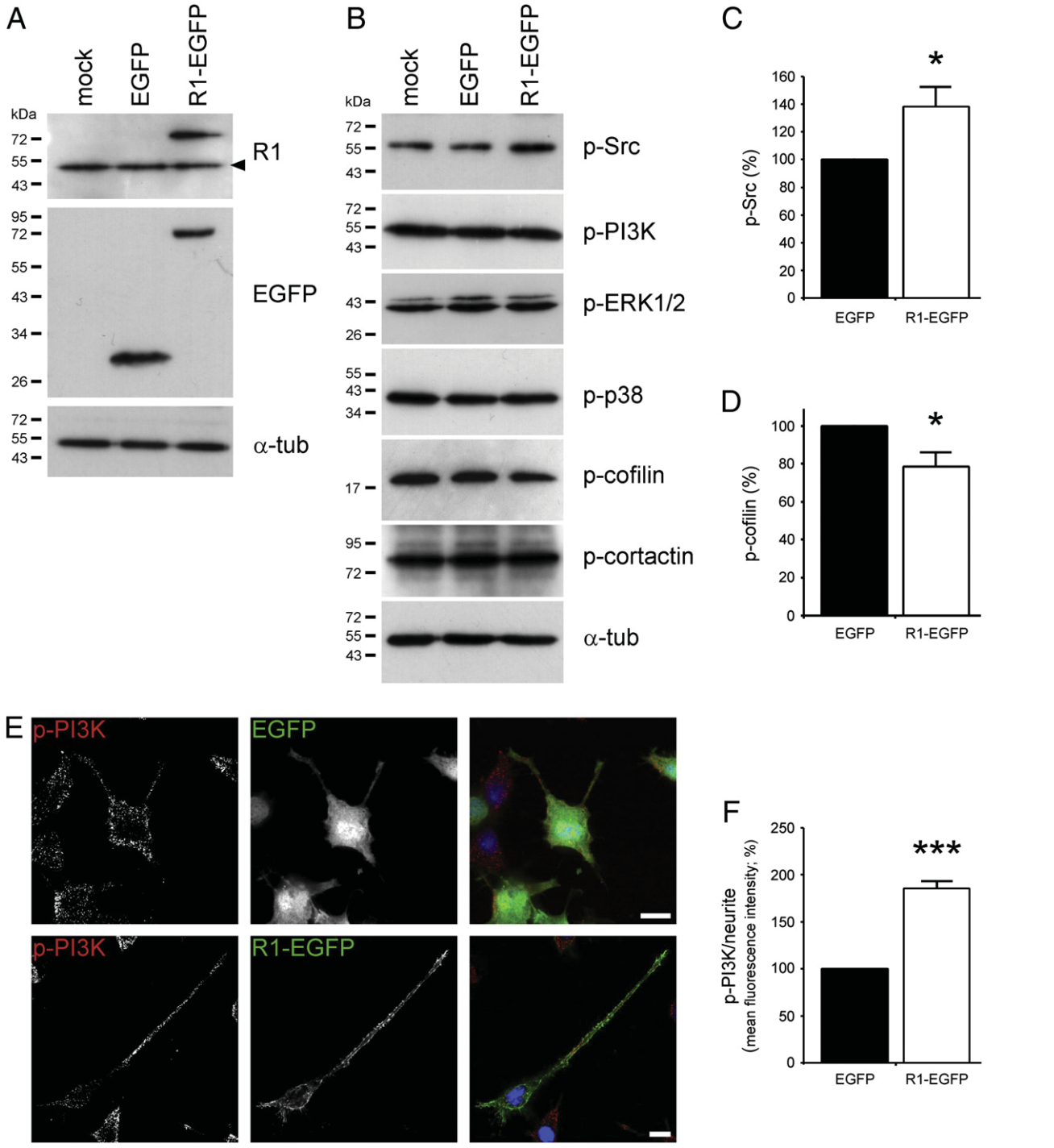

Fig. 6. Reggie-1 overexpression in N2a cells led to an activation of key signaling molecules of cytoskeleton remodeling. (A) Western blot analyses of cell extracts with Abs against reggie-1 (R1) and EGFP showed that both EGFP and reggie-1-EGFP (R1-EGFP) constructs were efficiently expressed in N2a cells. The black arrowhead indicates endogenous reggie-1. An Ab against alpha-tubulin (a-tub) served as loading control. (B) Abs against the phosphorylated forms of Src kinase (p-Src) and cofilin (p-cofilin) revealed their increased activation in R1-EGFP overexpressing as compared to mock and control EGFP transfected N2a cells. No significant difference was observed in the activation (phosphorylation) of PI3K (but see Fig. 7), ERK1/2, p38, and cortactin. (C,D) Quantification of the blots shown in B for p-Src (C) and p-cofilin (D) of R1-EGFP-transfected cells as percent of control EGFP-transfected cells. (E,F) p-PI3K immunofluorescence intensity was determined over the neurites of R1-EGFP and EGFP control N2a cells and showed an almost 100% increase over controls. Student's *t*-test; ***: $p<0.001$, **: $p<0.01$, *: $p<0.05$. Error bars, SEM. Scale bars, 10 μm.

Inhibition of ROCK further increased the growth promoting effect of reggie 1.

Reggie 1 overexpression in hippocampal neurons and N2a cells leads to an activation of key signaling molecules of cytoskeleton remodeling

To further characterize the regulation of possible effector molecules involved in the signaling of reggie 1 leading to enhanced neurite growth, we determined the effect of R1 EGFP overexpression on key signaling molecules of cytoskeleton remodeling in immunoblots and immunostainings of N2a and hippocampal neurons. In N2a cells, quantitative immunoblots showed a significant increase in p Src and decrease in p cofilin after transfection with R1 EGFP compared to con trol, while there was no significant change in the levels of p p38, p ERK1/2, p cortactin and p PI3K (Fig. 6). Since our pharmacological blockage experiments in hippocampal neurons, however, suggested that PI3K lies in the reggie 1 signaling pathway and since the immuno blots represent whole cell lysates, we examined whether an increase of p PI3K might be detected specifically in the neurites and growth cones of reggie 1 overexpressing neurons. Indeed, the intensity of immunostainings with anti p PI3K was significantly increased in N2a neurites and in hippocampal growth cones (Figs. 6E, F and 7). Hippocam pal growth cones also showed a significantly increased immunostaining

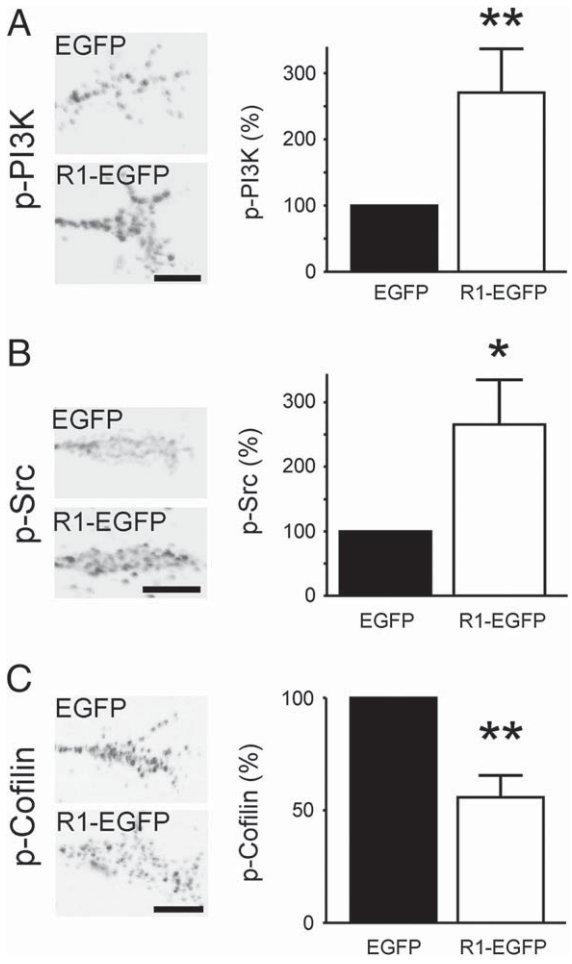

Fig. 7. Reggie-1 overexpression in hippocampal neurons led to an activation of key signaling molecules of cytoskeleton remodeling. Reggie-1-EGFP (R1-EGFP) overexpressing as compared to EGFP expressing control mouse hippocampal growth cones showed increased staining intensity for the phosphorylated (p) forms of (A) PI3K and (B) Src and resulted in (C) reduced staining intensity for p-cofilin (i.e., activation), as quantified in the corresponding histograms. Error bars, SEM, Student's *t*-test, *: $p<0.05$, **: $p<0.01$. Scale bars, 5 μm.

intensity for p Src and decreased staining for p cofilin consistent with the immunoblot results in N2a cells.

Together, these data show that overexpression of reggie 1 results in an increased activation of PI3K, Src tyrosine kinase as well as cofilin, particularly in the neurites and axonal growth cones. Thus, reggie 1 communicates with molecules which participate in the reg ulation of actin cytoskeleton dynamics and cargo supply of the growth cone, and its upregulation enhances growth.

Discussion

The reggie proteins function as key regulators of axon growth and regeneration in the fish visual pathway where both are upregulated in RGCs upon lesion (Munderloh et al., 2009). The present study has investigated if reggie 1 upregulation has beneficial effects for axon growth and regeneration in mature mammalian neurons that fail to spontaneously re express reggie. We show that upregulation of reggie 1 in rat RGCs in vivo indeed is sufficient to promote axon re generation in the lesioned optic nerve. Upregulation of reggie 1 im proves the neuron intrinsic conditions of RGCs to such an extent that a significant number of regenerating axons crossed the lesion site and advanced far into the distal portion of the adult optic nerve despite its non permissive properties. This growth promoting effect of reggie 1 is also found in mouse hippocampal and N2a neurons where it was technically feasible to relate increased neurite length to the activation of key signaling molecules and effectors of actin cy toskeletal dynamics, such as Src and PI3K, Rho GTPases, namely Rac1 and TC10, and their downstream effector cofilin.

Thus, reggie upregulation activates the cell intrinsic program that is required for axon regeneration. Moreover, it apparently provides to the neurons the ability to extend axons into the optic nerve despite the inhibitory environment in vivo and allows AAV.R1 EGFP trans duced RGCs in vitro to grow longer neurites on the most relevant in hibitory components of the optic nerve, CSPG and Nogo A. These findings are consistent with the notion that the growth cone inte grates over growth promoting and inhibiting signals and overcomes inhibitors when their influence is counterbalanced by intrinsic signal ing favoring growth cone elongation (Schwab, 2010).

The delta20 fragment of Nogo A inhibits neurite outgrowth across cell types and species (Abdesselem et al., 2009; Niederost et al., 1999; Oertle et al., 2003) through the activation of the Rho/ROCK/LIMK path way which finally targets cofilin to exert growth inhibition. Reggie controlled signals also target effectors of actin dynamics including cofilin (Munderloh et al., 2009) but in the opposite direction. Thus, the reggie mediated activation of cofilin can account for RGC axon growth in the presence of Nogo A and CSPG, and for the increased neurite length in both primary hippocampal neurons and N2a cells. Compared to earlier studies where cofilin activation (de phosphorylation) by inhi bition of RhoA and ROCK enhanced neuronal regeneration in vivo and in vitro (Lingor et al., 2007, 2008), the present pro regenerative effect of reggie 1 in RGCs was stronger than the former Rho/ROCK inhibition. In fact, reggies regulate a broader range of GTPases and seem to control membrane trafficking and the targeted delivery of cargo to the growth cone (Bodrikov et al., 2011; Stuermer, 2010) which is required for growth cone elongation. This complies with the fact that reggie upregulation in mouse hippocampal neurons led to 60% longer neurites than controls, and this gain in length was lost by the DN form of TC10 and by the PI3K blocker which affects the GTPase Rac1.

The GTPases TC10 and RalA are known to participate in the exocyst dependent cargo transport and delivery of bulk membrane and membrane proteins to the growth cone in neurons (Das and Guo, 2011; Dupraz et al., 2009). Consistently, reggie downregulation led to the inhibition of axon regeneration and blockage of neurite for mation in hippocampal neurons and adult fish RGCs (Munderloh et al., 2009). The present findings suggest that the reggie dependent ac tivation of signaling molecules, their influence on actin cytoskeletal

dynamics and function in the targeted delivery of cargo are crucial for axon regeneration in rat RGCs and possibly for CNS axon regrowth in general.

Although AAV.R1 EGFP enabled RGCs to extend long axons, their number was small. It is possible that reggie 1 upregulation might allow regeneration of a higher number of axons if the transduction ef ficiency across RGCs could be improved and the reggie 1 concentra tion in individual RGCs be increased. Moreover, axotomized RGCs are lost by apoptosis (Berkelaar et al., 1994) so that by 2 weeks after ONC only the remaining 10% of the original RGCs can regrow an axon. A combination of reggie 1 upregulation and inhibition of apoptosis or axonal degeneration may be the treatment of choice to increase the number of regenerating axons. A combination of treat ments including de repression of PI3K signaling through PTEN dele tion and RGC stimulation by growth factors (oncomodulin) and elevation of cAMP has recently led to RGC axon regeneration and the partial recovery of visual responses in mice (Benowitz and Yin, 2007; de Lima et al., 2012). The simultaneous upregulation of reggie 1 and 2 might further improve regeneration and become use ful for a therapeutic approach aiming at the functional restoration of vision. Dangerous side effects such as tumor growth in the transduced cells were not observed in this study.

Why mammalian RGCs, in contrast to the fish, do not spontane ously upregulate growth associated genes, such as reggie 1 and 2, remains an open question but suggests that the identification of the relevant gene regulatory factors in fish and mammals might turn out useful for future therapeutic interventions.

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