

ROCK inhibition and CNTF interact on intrinsic signalling pathways and differentially regulate survival and regeneration in retinal ganglion cells

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Functional regeneration in the CNS is limited by lesion-induced neuronal apoptosis and an environment inhibiting axonal elongation. A principal, yet unresolved question is the interaction between these two major factors. We thus evaluated the role of pharmacological inhibition of rho kinase (ROCK), a key mediator of myelin-derived axonal growth inhibition and CNTF, a potent neurotrophic factor for retinal ganglion cells (RGC), in models of retinal ganglion cell apoptosis and neurite outgrowth/regeneration *in vitro* and *in vivo*. Here, we show for the first time that the ROCK inhibitor Y-27632 significantly enhanced survival of RGC *in vitro* and *in vivo*. *In vitro*, the co-application of CNTF and Y-27632 potentiated the effect of either substance alone. ROCK inhibition resulted in the activation of the intrinsic MAPK pathway, and the combination of CNTF and Y-27632 resulted in even more pronounced MAPK activation. While CNTF also induced STAT3 phosphorylation, the additional application of ROCK inhibitor surprisingly diminished the effects of CNTF on STAT3 phosphorylation. ROCK activity was also decreased in an additive manner by both substances. *In vivo*, both CNTF and Y-27632 enhanced regeneration of RGC into the non-permissive optic nerve crush model and additive effects were observed after combination treatment. Further evaluation using specific inhibitors delineate STAT3 as a negative regulator of neurite growth and positive regulator of cell survival, while MAPK and Akt support neurite growth. These results show that next to neurotrophic factors ROCK inhibition by Y-27632 potently supports survival of lesioned adult CNS neurons. Co-administration of CNTF and Y-27632 results in additive effects on neurite outgrowth and regeneration. The interaction of intracellular signalling pathways may, however, attenuate more pronounced synergy and has to be taken into account for future treatment strategies.

Keywords: retinal ganglion cells; CNTF; rho kinase; axotomy; regeneration

Abbreviations: ANOVA = analysis of variance; BSA = bovine serum albumin; CNTF = ciliary neurotrophic factor; CTB = cholera toxin subunit-B; DAPI = 4,6-diamidino-2-phenylindole; DMEM = Dulbecco's modified Eagle's medium; EBSS = Earle's balanced salt solution; FCS = fetal calf serum; GAP = growth associated protein; HRP = horse radish peroxidase; JAK = janus kinase; MAG = myelin-associated glycoprotein; MAPK = mitogen-activated protein kinase; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; OMgp = oligodendrocyte-myelin glycoprotein; PBS = phosphate-buffered saline; PFA = paraformaldehyde; PKC = protein kinase C; PKN = protein kinase N; RGC = retinal ganglion cell; ROCK = rho kinase; SDS-PAGE = sodium dodecyl sulphate-polyacrylamide gel electrophoresis; STAT = signal transducer and activator of transcription

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Introduction

Spontaneous regeneration of CNS neurons after traumatic lesion or degenerative demise is limited and this accounts for the persistence of functional deficits in a variety of neurological disorders, e.g. spinal cord injury. Myelin-derived inhibitory molecules (i.e. Nogo, OMgp, MAG), as well as components of the glial scar (i.e. chondroitin sulphate proteoglycans) form a hostile environment, which prevents regenerating axons from re-establishing functional connections (Fawcett, 2006). Members of axonal guidance cue families, such as semaphorins or ephrins have equally been shown to act as inhibitory substrates in paradigms of CNS regeneration (Goldshmit *et al.*, 2004; Benson *et al.*, 2005). Myelin-derived inhibitors and guidance molecules bind to their different specific receptors but their signalling finally converges on the Rho/rho kinase (ROCK) pathway, rendering this pathway a promising target for pharmacological intervention in a restorative approach. Targeting the small GTPase Rho has been shown to increase regeneration in models of optic nerve lesion (Bertrand *et al.*, 2005) and pharmacological inhibition of ROCK dose-dependently increases the regeneration of retinal ganglion cells (RGCs) after optic nerve crush (Lingor *et al.*, 2007).

Apart from environmental factors, the intrinsic regenerative capacity of adult CNS neurons is reduced in comparison to peripheral or embryonic neurons, which is partially reflected in a differential regulation of regeneration associated genes, such as c-Jun or GAP43 (Schreyer and Skene, 1993; Schaden *et al.*, 1994; Broude *et al.*, 1997). In addition, the responsiveness of CNS neurons towards trophic stimuli derived from growth factors also decreases with the age of the neurons (Avwenagha *et al.*, 2003). Upon axonal lesion CNS neurons undergo a protracted apoptotic cell death which in addition limits the number of neurons capable of re-establishing functional connections (Berkelaar *et al.*, 1994). Ciliary neurotrophic factor (CNTF) is a potent trophic factor for RGCs and has been shown to rescue RGC from axotomy-induced apoptosis via activation of the JAK/STAT3 pathway (Ip *et al.*, 1993; Mey and Thanos, 1993). In models of neuroregeneration, CNTF promoted the outgrowth of RGC axons (Cui and Harvey, 2000), an effect which could be potentiated by elevated intracellular cAMP levels (Meyer-Franke *et al.*, 1995; Cui *et al.*, 2003). In addition, CNTF has been shown to protect RGCs in models of ocular hypertension (Ji *et al.*, 2004) and acute autoimmune optic neuritis in rats (Maier *et al.*, 2004).

A combinatorial strategy targeting inhibitory signalling pathways, increasing the intrinsic regenerative ability of adult neurons and interfering with lesion-induced cell death cascades thus seems promising to protect neuronal cell numbers and foster axonal regeneration in a restorative approach. In the present study, we examined whether pharmacological inhibition of ROCK by Y-27632, the application of CNTF or the combination of both may improve survival and regeneration of RGCs *in vitro* and

in vivo and which intracellular survival pathways are triggered by each substance alone or by their interactions. Our results show that the theoretical distinction between neuronal survival, afforded by neurotrophic factors, and axonal elongation, regulated by the Rho/ROCK pathway, cannot be maintained in a strict sense, since both parameters are regulated in an interactive manner. We show, that in addition to predicted synergism, the combination of both strategies may also result in the interference of cell signalling pathways and partially attenuate stronger synergistic effects.

Materials and Methods

RGC-5 cell culture, survival and outgrowth assays

RGC-5 cells (kind gift of Dr Krishnamoorthy, Department of Cell Biology and Genetics, University of North Texas Health Science Center, Fort Worth, TX, USA) were cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, Eggenstein, Germany) containing 10% fetal calf serum (FCS) and antibiotics (penicillin, streptomycin; Sigma-Aldrich, Munich, Germany) in a humidified atmosphere at 37°C with 5% CO₂ (Krishnamoorthy *et al.*, 2001).

For cell-survival experiments, RGC-5 cells were washed three times in Eagle's balanced salt solution (EBSS; Gibco) directly after trypsinization. Cells were then plated at a density of 500 cells/cm² in serum-free medium supplemented with succinyl concanavalin A (sConA; 50 µg/ml; Sigma-Aldrich) in order to induce cell differentiation as previously described (Seigel and Notter, 1992). The cell culture was kept serum-free for 6 days without any further supplementation (control), with the ROCK inhibitor Y-27632 (final concentration 10 µM; Calbiochem, Schwalbach, Germany), with recombinant rat CNTF (final concentration 50 ng/ml, PeproTech Inc., Rocky Hill, USA) or with a combination of Y-27632 and CNTF. Y-27632 and CNTF were added to the culture on Day 1 after plating and again supplemented at Day 4 *in vitro*. For survival experiments involving STAT3-, MAPK- or Akt inhibitors, cells were kept in regular medium with full serum supplementation. In order to assess cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Sigma-Aldrich) was added to the cells in a final concentration of 500 µg/ml and incubated at 37°C for 1 h. MTT-positive cells indicative for viability were counted in six random fields within a culture well. In addition, cell death was quantified after nuclear staining with DAPI (4,6-diamidino-2-phenylindole) (Sigma-Aldrich). The number of cells with fragmented nuclei was set in relation to total cell number in culture and this ratio was given as per cent of apoptotic cells.

For neurite outgrowth assays, cells were kept in regular culture medium because serum withdrawal was found to be severely inhibiting cell survival. Y-27632 and CNTF were added in the same final concentrations as mentioned before.

Photographs of four random visual fields per well were taken after 2 days in culture and neurite length was evaluated semi-automatically using the axon tracing module of 'Image J' (Free Java software provided by the National Institutes of Health, Bethesda, MD, USA). Inhibitors of STAT3 (AG 490; 50 µM), MAPK (PD 98059, 50 µM) and Akt (Triciribine, 2 µM), all from Calbiochem, Schwalbach, Germany, were applied at the same

time as Y-27632 and CNTF in neurite outgrowth and cell-death experiments.

All cell culture experiments were performed in triplicate and repeated at least three times.

Primary RGC culture, survival and outgrowth assays

For immunopurified rat RGC cultures, Wistar rat pups were sacrificed on postnatal Day 7–8. RGCs were purified according to a two-step panning protocol for Thy-1 to >99.5% purity as described previously (Barres *et al.*, 1988). Cells were cultured in serum-free neurobasal medium (Gibco), supplemented with B-27 supplement, pyruvate (Sigma-Aldrich), glutamine, cysteine, triiodothyronine, Sato (BSA, transferrin, progesterone, putrescine, sodium selenite) (Gibco), forskolin (final concentration 10 μ M), human BDNF (final concentration 50 ng/ml) (Tebu, Offenbach, Germany), insulin (final concentration 5 μ g/ml) (Sigma-Aldrich) and CNTF (final concentration 10 ng/ml) (Tebu). Cell cultures used for growth factor deprivation studies were initially plated in full medium after preparation and then were switched 24 h after plating to medium as described in the following paragraphs.

For survival assays primary RGC were plated at a density of 5000/well on 96well-plates (Sarstedt, Nümbrecht, Germany) coated with poly-d-lysine. After incubation for 24 h in full medium the cells were deprived of neurotrophins by medium change and cultures were supplemented with ROCK inhibitor Y-27632 (final concentration 10 μ M), CNTF (final concentration 50 ng/ml) or both. Viability was assessed after 2 days by counting MTT-positive RGC in six random microscopic fields within a culture well after addition of MTT (final concentration 500 μ g/ml) and incubation at 37°C for 1 h.

For outgrowth assays, cells were first grown in full culture medium for 1 day and then withdrawn only from CNTF because deprivation of all neurotrophins resulted in a severely reduced cell survival rate. Neurite elongation was evaluated at Day 3 *in vitro* after 2 days of treatment with Y-27632 (final concentration of 10 μ M) and/or recombinant rat CNTF (final concentration of 50 ng/ml). Photographs of four random visual fields per culture well were taken and neurite length was evaluated semi-automatically using the axon tracing module of 'Image J' (National Institutes of Health, Bethesda, MD, USA). All cell culture experiments have been performed in triplicate and repeated at least three times.

Axotomy of the optic nerve, optic nerve crush and peripheral nerve graft

All animal experiments were carried out according to the regulations of the local animal research council and legislation of the State of Lower Saxony. In all animal experiments adult female Wistar rats (200–250 g; Charles River, Sulzfeld, Germany) were used.

The anaesthesia was carried out by intraperitoneal injection of chloral hydrate (420 mg/kg body weight). A similar surgical approach was used in all three experimental paradigms to access the optic nerve: the skin was incised close to the superior orbital rim and the orbita was opened leaving the supraorbital vein intact. The intraorbital glands were moved aside and the superior extraocular muscles detached from their tendinous insertion points. The eye was then rotated in the ventral direction and

the optic nerve exposed by longitudinal incision of the optic nerve sheath.

For axotomy experiments, the optic nerve was then transected ~2 mm from the posterior eye pole taking care not to damage the retinal blood supply. A 2 \times 2 mm piece of gel foam (Braun, Melsungen, Germany) was soaked in FluoroGold (Hydroxystilbamidine; Bio-Trend, Cologne, Germany) and placed on the optic nerve stump in order to retrogradely label RGC. On Day 14 post-axotomy, animals were sacrificed by CO₂ inhalation and the eyes were extracted. The cornea, the lens and the vitreous body were removed, and the remaining eye cup containing the retina was fixed in 4% paraformaldehyde in PBS, pH 7.4 (PFA) for 1 h. Retinae were then extracted and flat-mounted in glycerol-PBS (1:1) on glass slides. The number of FluoroGold-positive RGC was determined by fluorescence microscopy (Zeiss-Axioplan, Oberkochen, Germany) using a UV filter (365/420 nm). Three fields of 62 500 μ m² were counted in each retinal quadrant (at eccentricities of one-sixth, one-half and five-sixths of the retinal radius). RGC counts were performed independently by two different investigators according to a blinded protocol. Axotomy groups consisted of the following animal numbers: PBS: $n = 4$, Y-27632: $n = 6$, CNTF: $n = 5$, combination: $n = 6$.

For optic nerve crush experiments, the optic nerve was ligated using a 10/0 suture (Ethicon, Johnson–Johnson, Livingston, UK) for 30 s, resulting in a complete transection of all RGC axons. The suture was removed and the operative access closed. Rats were sacrificed on Day 28 of the study by CO₂ inhalation and immediately perfused by transcardial injection of 250 ml PBS and 200 ml 4% PFA. The eye and the optic nerve were removed *en bloc* from the orbit. The cornea, the lens and the vitreous body were removed and the remaining eye cup containing the retina and the adjacent optic nerve were post-fixed in 4% PFA for 1 h. The tissue was then dehydrated in 30% sucrose overnight and kept at –20°C until further processing. Longitudinal sections (16 μ m) of the optic nerve were prepared using a Leica cryostat and collected on gelatine-coated glass slides. For evaluation of RGC axon regeneration slides were immunostained for GAP-43 and photomicrographs were taken using a fluorescence microscope (Axiovert 35, Zeiss, Germany). The number of regenerating axons at designated distances from the crush was evaluated using a counting grid superimposed on the photomicrograph. Optic nerve crush groups consisted of the following animal numbers: PBS: $n = 7$, Y-27632: $n = 7$, CNTF: $n = 6$, combination: $n = 4$.

For peripheral nerve autograft experiments the optic nerve was transected ~2 mm from the posterior eye pole. A piece of 2.5 cm length of the sciatic nerve was dissected from the left leg and grafted to the optic nerve stump using a 10/0 suture. The distal part of the sciatic nerve was placed in an osseous canal milled in advance on the surface of the skull.

Labelling of regenerating axons was achieved by intravitreal injection of 3 μ l Cy3-labeled cholera toxin subunit-B (CTB; Molecular Probes, Eugene, Oregon) into the vitreous chamber 1 day before sacrificing the animal. Rats were sacrificed on Day 28 of the study by CO₂ inhalation and immediately perfused by transcardial injection of 250 ml PBS and 200 ml 4% PFA. The eye, the optic nerve and the peripheral nerve graft were removed *en bloc* from the orbit. The cornea, the lens and the vitreous body were removed and the remaining eye cup containing the retina, the adjacent optic nerve and the graft were post-fixed in 4% PFA for 1 h. The tissue was then dehydrated in 30% sucrose overnight

and kept at -20°C until further processing. Longitudinal sections of the optic nerve stump and the adjacent graft ($16\ \mu\text{m}$) were prepared using a Leica cryostat and collected on gelatine-coated glass slides. Evaluation of CTB fluorescence indicative for regenerating axons was performed directly from non-processed slides using a fluorescence microscope (Axiovert 35, Zeiss, Germany) and a filter for Cy3-fluorescence.

For quantification of regenerating axon numbers, seven regularly spaced sections in each graft were evaluated using a grid superimposed on the photomicrograph. Numbers of CTB-positive axons crossing perpendicular grid lines were counted at multiples of 1 mm from the grafting point until the end of the graft. For each section the sum of all counted axons was calculated and indicated as cumulative number of regenerating axons.

Peripheral nerve graft groups consisted of the following animal numbers: PBS: $n=6$, Y-27632: $n=5$, CNTF: $n=4$, combination: $n=5$.

After all surgical procedures retinal blood supply was verified by funduscopy and animals with persistent retinal ischaemia were excluded. All eyes were checked for cataracts induced by lens injury after repeated intravitreal injections and animals were excluded from the study in case of cataract formation.

Dosage and application of Y-27632 and CNTF for *in vivo* experiments

All intravitreal injections were performed using a glass microelectrode connected to a Hamilton precision syringe, puncturing the eye at the cornea–sclera junction. Care was taken to not puncture the lens. Vehicle or test substance measuring $3.5\ \mu\text{l}$ were injected at each injection time point. For ROCK inhibition, a 2.9 mM solution of Y-27632 in PBS was injected, resulting in an application of $\sim 3.3\ \mu\text{g}$ of Y-27632 per injection. The concentration of Y-27632 used in the present study was based on data from a previous study where we have evaluated the dose-dependent effects of Y-27632 on RGC regeneration (Lingor *et al.*, 2007). For CNTF injections $1.5\ \mu\text{g}$ of CNTF were administered in PBS. For combination treatments $\sim 3.3\ \mu\text{g}$ of Y-27632 and $1.5\ \mu\text{g}$ of CNTF were administered in $3.5\ \mu\text{l}$ PBS solution. In all experimental paradigms substance injections were performed three times: directly after surgery (Day 0), on Day 3 and Day 6 after surgery.

Western blots

RGC-5 cells were washed three times in EBSS after trypsinization and kept in serum-free medium supplemented with concanavalin A on Day 1 in culture. Lysates were prepared on Day 3 after 30 min and 240 min of treatment with the ROCK inhibitor Y-27632 (final concentration $10\ \mu\text{M}$), with recombinant rat CNTF (final concentration $50\ \text{ng/ml}$) or with a combination of Y-27632 and CNTF. For inhibition of MAPK signalling PD-98059 was added to the cell culture at a final concentration of $50\ \mu\text{M}$. The lysis buffer was composed of 10 mM HEPES (pH 7.2), 142 mM KCl, 5 mM MgCl_2 , 1 mM EGTA and 1% IGEPAL plus protease inhibitors ('Complete tablets', Roche). The protein content of the cell lysate samples was determined using the bicinchoninic acid assay (Pierce, Rockford, IL, USA) and equal amounts of protein ($20\ \mu\text{g}$) were loaded in each lane for a sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were then transferred to a nitrocellulose membrane and blocked with 5% BSA in Tris-buffered saline/Tween-20 (TBS-T)

for 1 h. Membranes were then incubated with primary antibodies (anti-phospho-STAT3 (1:1000), anti-STAT3 (1:1000), anti-phospho-Akt (1:1000), anti-Akt (1:1000), anti-phospho-MAPK (1:1000), anti-MAPK (1:1000), (all Cell Signaling Technologies, Danvers, MA, USA) anti-ROCK-II (Dilution 1:250), anti-RhoA (Dilution 1:500) (both Santa Cruz, Heidelberg, Germany) for 24 h at 4°C in TBS-T and 2% BSA or with anti-beta Tubulin (1:5000, Sigma, Munich, Germany) for 1 h at room temperature in TBS-T and 5% BSA. This was followed by incubation with corresponding horseradish peroxidase-coupled secondary antibodies (1:2000, for 1 h at room temperature; Dianova, Hamburg, Germany). ECL-Plus reagent (Amersham, Arlington Heights, IL, USA) was applied on the membrane and the chemiluminescence was visualized and quantified using a Fluor-S-max imager (Bio-Rad, Munich, Germany).

Immunohistochemistry

Retinal sections were dehumidified at 37°C for 1 h and antigen retrieval was performed for 4 h in TBS-T (pH 9.0) at 60°C . Unspecific binding was blocked by application of 10% new-born goat or horse serum, and primary antibodies (anti-ROCK-II, 1:250, sc-1851 (C20) Santa-Cruz; anti-GAP43, 1:500, Abcam) were applied at 4°C overnight. Secondary antibody (Alexa 546-labelled anti-goat, 1:200 or Cy2-labelled anti-rabbit; Dianova) was applied 1:300 for 45 min at room temperature. The sections were nuclear counter-stained with DAPI (4,6-diamidino-2-phenylindole) (Sigma-Aldrich) and mounted in Moviol (Hoechst, Frankfurt, Germany).

ROCK activity assay

Similar to western blot experiments, RGC-5 cells were cultured for 3 days in serum-free medium supplemented with concanavalin A on Day 1 and then treated for 30 or 240 min with the ROCK inhibitor Y-27632 (final concentration $10\ \mu\text{M}$), with recombinant rat CNTF (final concentration $50\ \text{ng/ml}$) or with a combination of Y-27632 and CNTF. The lysis buffer was composed of 20 mM Tris–HCl (pH 7.4), 1% Triton X-100 plus protease inhibitors ('Complete tablets', Roche). The protein content of the cell lysate samples was determined using the bicinchoninic acid assay (Pierce). The ROCK activity assay was performed according to the manufacturer's instructions (CycLex, Nagano, Japan). Briefly, equal amounts of protein (50 ng) were applied to the assay plate wells and incubated with ATP-containing kinase reaction buffer at 30°C for 30 min. After washing and incubation with the HRP, conjugated antibody detection was performed at room temperature for 60 min. After another washing step, the tetramethylbenzidine containing substrate reagent was added and incubated at room temperature for 10 min until the reaction was stopped with 0.5 N sulphuric acid. The absorbance was measured in a Tecan spectrophotometric plate reader (Tecan, Crailsheim, Germany) at a 450 nm single wavelength.

Statistics

All *in vitro* experiments and western blots were reproduced at least in triplicate. Animal groups were as indicated in the according methods section. Differences between groups were considered statistically significant according to a one-way ANOVA followed by a parametric multiple comparison test (Dunnett test).

Significances were indicated with $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, unless otherwise stated.

A comprehensive summary of all antibodies and inhibitors used in this study is given in Supplementary Table S1.

Results

ROCK inhibition potently increases RGC survival *in vitro*

In order to examine the effect of ROCK inhibition on the survival of RGCs, we employed models of serum-deprived RGC-5 cells and neurotrophin-deprived primary RGCs. We compared the effect of ROCK inhibition on cell survival with the application of a saturating amount of CNTF and the combination of both substances. After 6-day serum deprivation only $8.0\% \pm 1.4\%$ of the initial RGC-5 population were still viable (by MTT exclusion test) and $77.8\% \pm 2.8\%$ showed fragmented apoptotic nuclei (DAPI staining) in control cultures. Addition of the ROCK-inhibitor Y-27632 ($10 \mu\text{M}$) to the culture medium increased the number of MTT-positive cells and decreased the number of cells with fragmented nuclei ($25.3\% \pm 3.5\%$ and $53.8\% \pm 1.8\%$, respectively). CNTF substitution induced a similar effect ($27.4\% \pm 1.7\%$ and $53.1\% \pm 3.0\%$, respectively). The combination of both substances resulted in even more pronounced survival ($35.4\% \pm 2.5\%$ and $42.8\% \pm 2.4\%$, respectively), which was significantly higher than in cultures treated with either substance alone (Fig. 1A and B). Serum-deprived control cultures showed cells with retracted processes and a tendency to form aggregates. Cells treated with Y-27632 exhibited less pronounced morphological signs of degeneration, which was similar to CNTF-treated cultures. Cultures treated with the combination of Y-27632 and CNTF most closely resembled the non-serum deprived control, although numbers of surviving cells and apoptotic nuclei did not reach the equivalent numbers of the control group (Fig. 1C).

We compared the results obtained in the cell line with a primary culture of RGCs which were neurotrophin-deprived for induction of apoptotic cell death. In control cultures, only $33.5\% \pm 2.2\%$ of the cells were viable after 2 days in culture (by MTT exclusion test) and $62.0\% \pm 6.0\%$ showed fragmented apoptotic nuclei (DAPI staining). Similar to the results obtained in the RGC-5 cell line application of the ROCK inhibitor enhanced survival after neurotrophin deprivation ($36.8\% \pm 2.2\%$) the number of cells with fragmented nuclei was markedly decreased ($51.8\% \pm 2.0\%$). Supplementation with CNTF significantly increased survival of primary RGC neurons ($64.0\% \pm 8.3\%$) and decreased the fragmentation of nuclei ($44.6\% \pm 1.9\%$). The combination of CNTF and Y-27632 again resulted in increased survival and decreased nuclear fragmentation, although the additive effect of the combination treatment was less pronounced compared to RGC-5 cells ($69.1\% \pm 4.5\%$ and $41.6\% \pm 1.0\%$, respectively) (Fig. 2A–C).

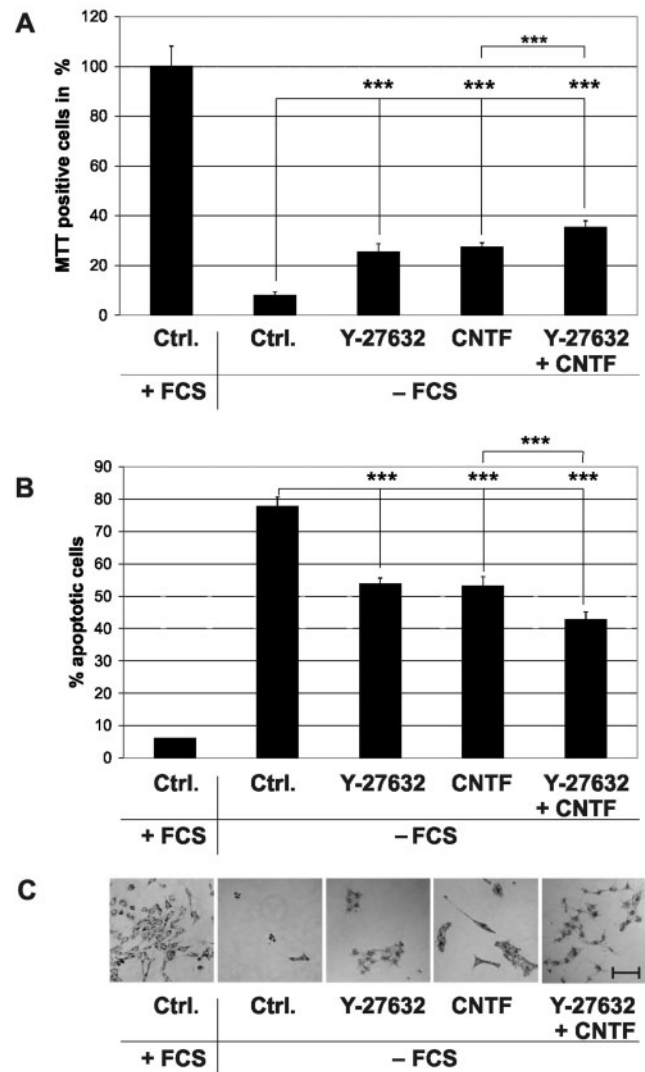


Fig. 1 ROCK inhibition and CNTF increase survival of RGC-5 cells after 6 days of serum deprivation. **(A)** Evaluation of cell survival by quantification of MTT-positive cells. ROCK inhibition by Y-27632 and CNTF significantly increase RGC-5 cell survival and the combination treatment is significantly more potent than either substance alone. **(B)** Numbers of apoptotic nuclei are decreased after treatment with CNTF, Y-27632 and the combination was significantly more potent than either substance alone. **(C)** Photomicrographs of representative RGC-5 cultures stained with MTT as depicted in **A**. $***P < 0.001$. Bar: $100 \mu\text{m}$.

ROCK inhibition and CNTF increase neurite outgrowth of RGC *in vitro*

We then evaluated, whether ROCK inhibition and CNTF treatment have a similar combinatorial effect on neurite outgrowth in RGC-5 cells and primary RGC cultures. Neurite length per cell was quantified on 2 days after substance application in RGC-5 cells and in primary RGC.

Due to the induction of considerable cell death in the RGC-5 culture after 2 days of serum deprivation, the culture was kept in full serum-containing medium.

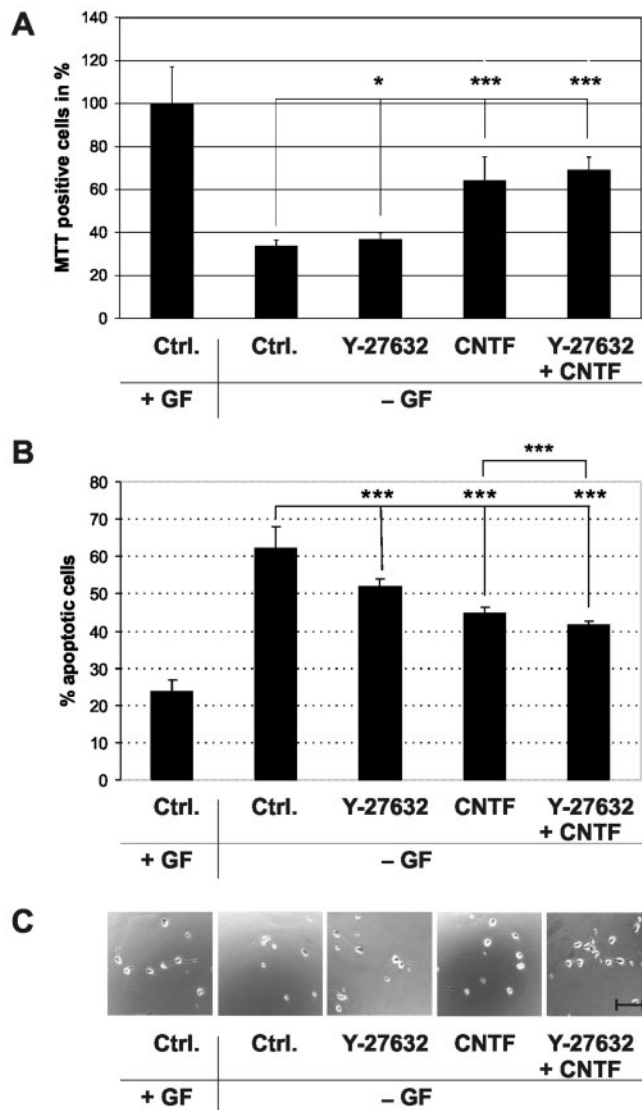


Fig. 2 ROCK inhibition and CNTF increase survival of growth factor-deprived primary retinal ganglion cells. **(A)** Survival after 2 days of growth factor deprivation evaluated by quantification of MTT-positive cells. Y-27632, CNTF and the combination of all significantly increased cell survival. **(B)** Decreased numbers of apoptotic nuclei after application of CNTF, Y-27632 or the combination of both. **(C)** Photomicrographs of primary RGC corresponding to the treatment groups as depicted in **A**. * $P < 0.05$; *** $P < 0.001$. Bar: 100 μm .

After 2 days of treatment with Y-27632, RGC-5 cells showed a significantly enhanced mean neurite outgrowth ($34.6 \mu\text{m} \pm 2.0 \mu\text{m}$) compared to non-treated RGC-5 cells ($24.2 \mu\text{m} \pm 1.8 \mu\text{m}$). Supplementation of the culture medium by CNTF showed also increased neurite lengths ($30.7 \mu\text{m} \pm 1.5 \mu\text{m}$). The combination treatment with Y-27632 and CNTF resulted in significantly greater length of outgrowing neurites compared to treatment with either substance alone ($49.1 \mu\text{m} \pm 3.8 \mu\text{m}$) (Fig. 3A and B).

Primary RGCs cultured in CNTF-free medium displayed a significantly increased neurite outgrowth after

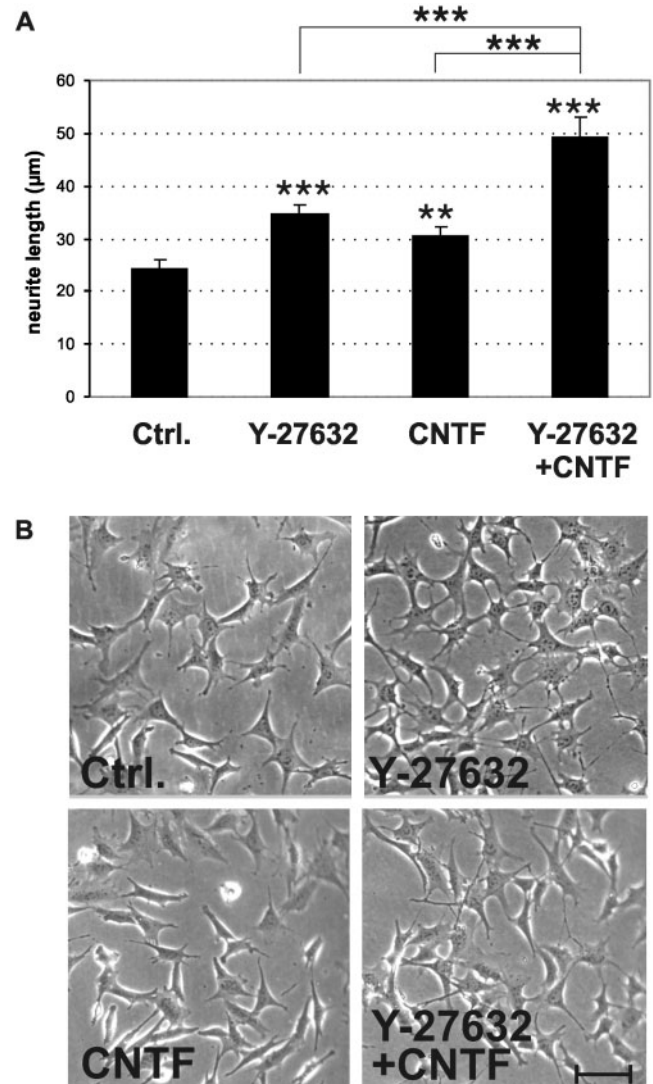


Fig. 3 ROCK inhibition and CNTF enhance neurite outgrowth in RGC-5 cells. **(A)** Evaluation of neurite outgrowth 48 h after application of Y-27632, CNTF and the combination. All treatments significantly increase neurite outgrowth. The dual treatment demonstrates an additive effect compared to the single treatment. ** $P < 0.01$; *** $P < 0.001$. **(B)** Phase contrast photomicrographs of representative cultures corresponding to the treatment groups as depicted in **A**. Bar: 50 μm .

supplementation with Y-27632 ($526.2 \mu\text{m} \pm 115.2 \mu\text{m}$) or CNTF ($489.1 \mu\text{m} \pm 83.0 \mu\text{m}$) as compared to control cells ($228.1 \mu\text{m} \pm 36.1 \mu\text{m}$). Supplementation of both Y-27632 and CNTF enhanced neurite outgrowth to an even larger extent ($600.6 \mu\text{m} \pm 100.9 \mu\text{m}$), though the additional effect was less pronounced than in the RGC-5 culture (Fig. 4A and B).

Regulation of intrinsic survival pathways by ROCK inhibition and CNTF

In order to elucidate the intracellular signalling cascades involved in the combinatorial effects of ROCK inhibition

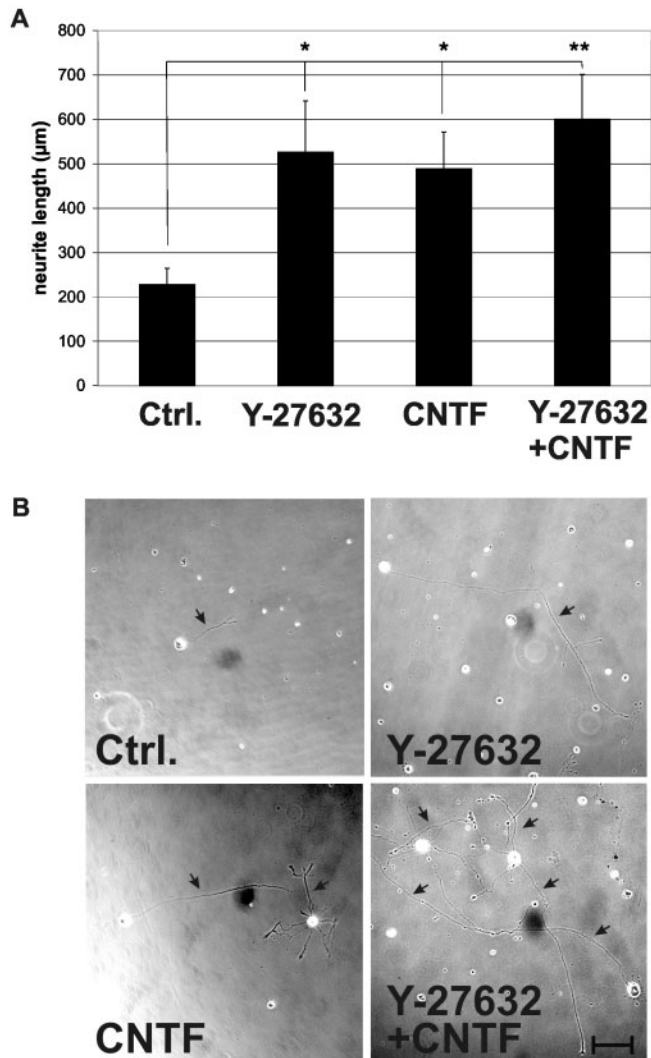


Fig. 4 ROCK inhibition and CNTF enhance neurite outgrowth in primary retinal ganglion cells. **(A)** Evaluation of neurite outgrowth 48 h after application of Y-27632, CNTF and the combination. All treatments significantly increased neurite outgrowth. * $P < 0.05$; ** $P < 0.01$. **(B)** Phase contrast photomicrographs of primary RGC cultures corresponding to the treatment groups as depicted in **A**. Arrows mark neurites. Bar: 100 μm .

and CNTF treatment on survival and neurite growth, we evaluated the protein levels of three canonical signalling pathways employed by numerous growth factors: the STAT3-, Akt- and MAPK cascades. RGC-5 cultures were treated with CNTF, Y-27632 or the combination of both substances and lysed 30 or 240 min after substance application. Changes in protein levels were determined by western blot quantification. We observed no marked changes in the protein levels of the non-phosphorylated STAT3, Akt or MAPK at any time point (Fig. 5A–G). Interestingly, both ROCK inhibition and CNTF application resulted in an increase of phospho-MAPK levels 30 min after treatment, which was even more pronounced after application of both substances (Fig. 5A and D). Treatment of cultures with the MAPK inhibitor PD 98059 (50 μM)

abolished MAPK phosphorylation in all treatment groups (data not shown). We thus suggest that the survival promoting effect of ROCK inhibition is mediated via phospho-MAPK. Interestingly, this pathway is regulated in a synergistic manner by a combined application of CNTF and Y-27632. Addition of CNTF resulted in increased phosphorylation of STAT3 30 min after application. While ROCK inhibition alone did not alter phospho-STAT3 levels, the combination of ROCK inhibition and CNTF treatment resulted in lower levels of phospho-STAT3 compared to cultures treated only with CNTF (Fig. 5A and B). This suggests that ROCK inhibition interferes with STAT3 signalling by CNTF and therefore attenuates its effects. Levels of phospho-Akt showed a trend towards upregulation 30 min after combination treatment ($P = 0.08$), equally suggesting a synergistic activation of the Akt pathway by ROCK inhibition and CNTF (Fig. 5A and F). No significant changes in phosphorylated and non-phosphorylated protein levels were detected at the 240 min time point (Fig. 5A, C, E and G).

Regulation of RhoA/ROCK levels and ROCK activity by ROCK inhibition and CNTF

We next examined the regulation of RhoA and ROCK-II levels in Y-27632, CNTF and combination-treated cultures. Both, after 30 and after 240 min no significant changes in RhoA or ROCK-II levels could be detected, which argues against a modulation of these two members of the inhibitory cascade in this paradigm (Fig. 5A, H–K). Since ROCK expression levels *per se* do not allow to draw conclusions about the enzymatic activity of ROCK, which is pivotal for downstream inhibitory signalling, ROCK activity levels were measured using an enzymatic ELISA-assay. At the early time point (30 min after substance application) no significant changes in ROCK activity were detected. Interestingly, at 240 min after substance application, Y-27632 and CNTF both showed a reduction in ROCK activity and the combination of both substances resulted in a more pronounced decrease in enzymatic activity to $\sim 65\%$ of control values (Fig. 5L).

Differential roles for STAT3-, MAPK- and Akt signalling in neurite elongation and survival

To more precisely dissect the roles of the signalling pathways, which are regulated by CNTF and ROCK inhibition, we evaluated neurite length and cell survival in RGC-5 cells (in full medium) under the influence of small molecule inhibitors of STAT3 (AG 490), MAPK (PD 98059) and Akt (Triciribine).

Inhibition of STAT3 by AG 490 resulted in an increased neurite outgrowth in PBS-, Y-27632- and combination-treated cells compared to respective cultures without

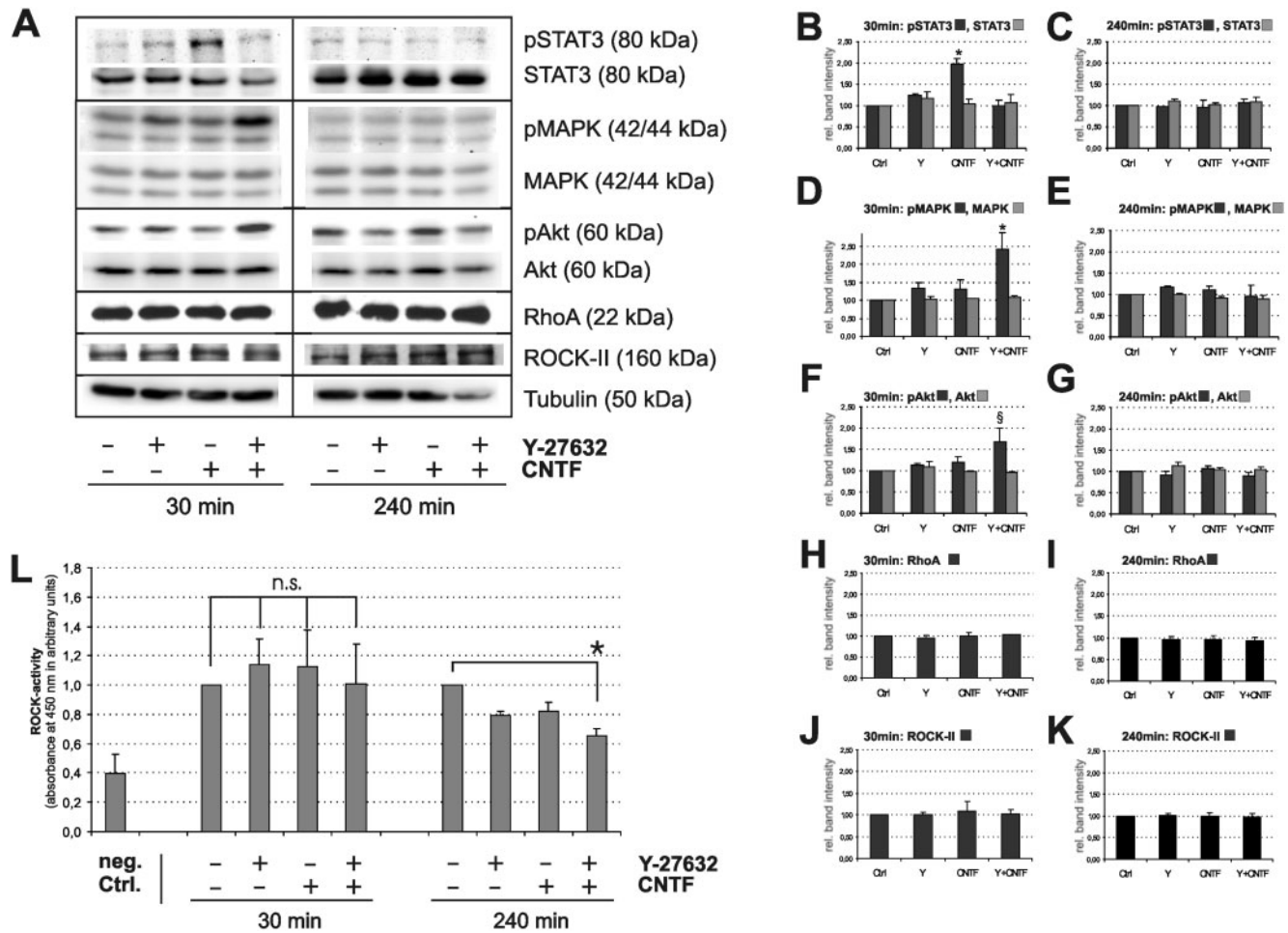


Fig. 5 Regulation of STAT3-, MAPK- and Akt-pathways, RhoA and ROCK-II in RGC-5 cells. **(A)** Representative western blots showing the regulation of STAT3, pSTAT3, MAPK, pMAPK, Akt, pAkt, RhoA and ROCK-II after application of Y-27632, CNTF or the combination of both. CNTF application results in increased phosphorylation of STAT3 after 30 min. This effect is abolished after application of the combination of CNTF and Y-27632. Both CNTF and Y-27632 result in increased phosphorylation of MAPK 30 min after application and this effect is increased by the combination treatment. Akt showed a trend towards increased phosphorylation in the combination treatment (§ $P = 0.08$). Protein levels of non-phosphorylated proteins did not differ substantially in all treatment groups. No significant changes were observed 240 min after substance application. Levels of RhoA or ROCK-II did not show any significant differences at any time point. **(B–G)** Quantification of Western blot bands from three independent experiments for STAT3/pSTAT3 **(B, C)**, MAPK/pMAPK **(D, E)**, Akt/pAkt **(F, G)**, RhoA **(H, I)** and ROCK-II **(J, K)** at 30 min **(B, D, F, H, J)** and 240 min **(C, E, G, I, K)** after substance application. ROCK activity (measured by ROCK activity ELISA) after application of Y-27632, CNTF or the combination of both after 30 and 240 min. A significant downregulation of ROCK activity is observed after the combination treatment 240 min after substance application. Negative control (Neg. Ctrl.) refers to untreated control lysate supplemented with Y-27632 (200 nM) directly before the assay **(L)**. * $P < 0.05$.

inhibitor treatment. In contrast, MAPK inhibition by PD 98059 resulted in a reduction of neurite length in comparison to no inhibitor treatment, which was most prominent in the Y-27632-treated group and both groups receiving CNTF. Akt inhibition by triciribine even more dramatically reduced neurite outgrowth in all groups compared to cells without inhibitor application (Fig. 6A). In contrast, survival of RGCs in full medium was markedly decreased by application of the STAT3 inhibitor AG 490 and the Akt inhibitor triciribine, while application of the MAPK inhibitor PD 98059 did not markedly alter survival of RGC-5 cells in full medium (Fig. 6B).

ROCK-II expression is upregulated in the retina following optic nerve transection

In vitro, inhibition of ROCK by Y-27632 showed beneficial effects on cell survival and neurite outgrowth and thus suggested a negative role for ROCK in the regulation of these events. To determine the importance of ROCK in traumatic injury of the optic nerve, we examined the expression of ROCK-II in the inner retinal layers by immunohistochemistry at different time points after axotomy. Untreated retinae showed no visible ROCK-II immunofluorescence. Transection of the optic nerve induced an increase of ROCK-II expression in the RGC

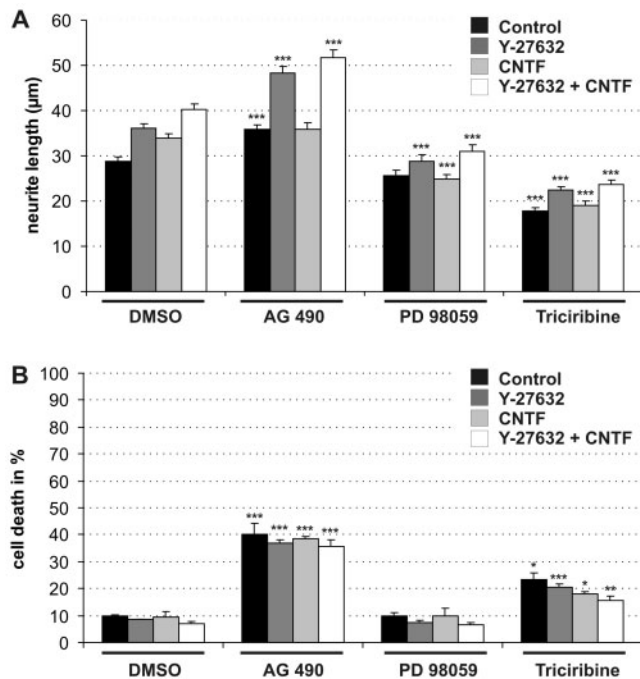


Fig. 6 Neurite outgrowth (**A**) and cell death in percent of apoptotic cells (**B**) in RGC-5 cultures treated with Y-27632, CNTF or both under the influence of small molecule inhibitors for STAT3 (AG 490, 50 µM), MAPK (PD 98059, 50 µM) and Akt (Triciribine, 2 µM). Cultures were kept in full medium. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ as compared to respective DMSO-treated controls.

layer starting at Day 1 and peaking at Day 4 following the transection of the optic nerve (Fig. 7A). At Day 7 post axotomy, the immunoreactivity for ROCK-II was declining again (data not shown). The early axotomy-induced upregulation of ROCK-II in the RGC layer is suggestive for an acute response of the lesioned neurons, which is likely to contribute to a post-lesional inhibition of the regenerative response and cell death.

ROCK inhibition and CNTF increase RGC survival following axotomy

RGCs undergo a well-characterized apoptotic cell death following transection of the optic nerve (Bahr, 2000). Based on the data demonstrating protection of RGC *in vitro*, we now evaluated the effect of intravitreal application of Y-27632 and CNTF on the survival of RGCs following complete transection of the optic nerve. All substances were applied at the day of axotomy and subsequently on Days 3 and 6. RGCs were retrogradely labelled by administration of FluoroGold during the axotomy procedure and surviving RGCs were counted at Day 14 post-axotomy in retinal flat mounts. RGC numbers in control animals receiving an intravitreal injection of PBS were set to 100%. ROCK inhibition by Y-27632 (3.3 µg in 3.5 µl)

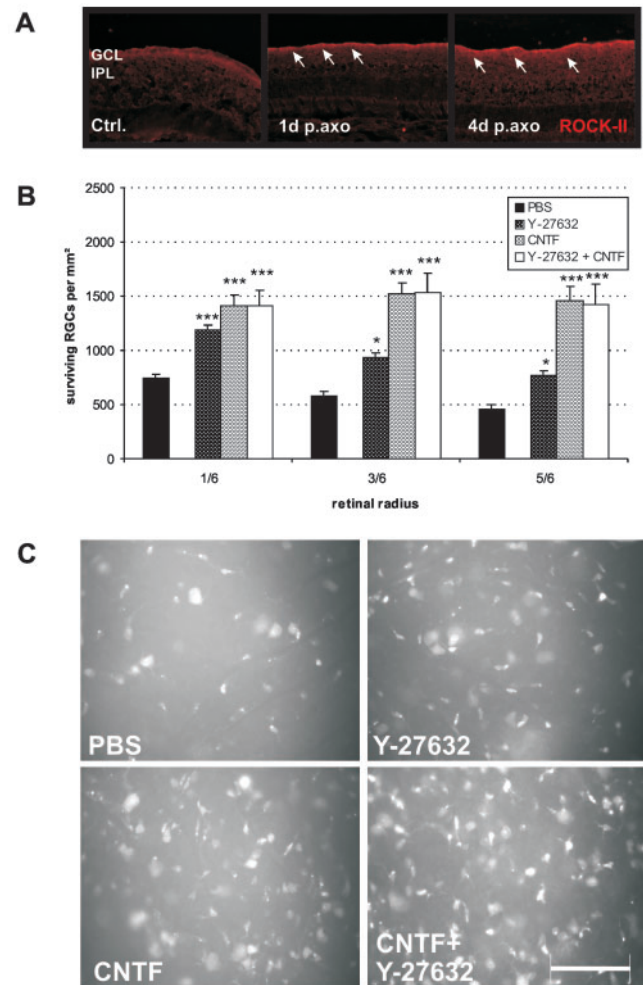


Fig. 7 ROCK inhibition and CNTF promote survival of RGC after axotomy. (**A**) Time course of ROCK-II expression in the inner retinal layers following transection of the optic nerve (GCL = ganglion cell layer, IPL = inner plexiform layer). Photomicrographs of non-axotomized control retina and retinae at 1 and 4 days post axotomy immunostained for ROCK-II. Arrows point out retinal ganglion cells in the GCL. (**B**) Intravitreal application of Y-27632, CNTF and a combination of both substances increase the survival of axotomized retinal ganglion cells in the inner, middle and outer retinal radii compared to PBS-treated animals. * $P < 0.05$; *** $P < 0.001$. (**C**) Photomicrographs of retinal flat mounts of representative middle retinal radii. RGCs are retrogradely labelled by FluoroGold. Bar: 200 µm.

resulted in $160 \pm 7\%$, $161 \pm 7\%$ and $166 \pm 10\%$ of surviving RGCs in the inner, middle and outer retinal radii, respectively, as compared to PBS-treated retinae. Application of CNTF (1.5 µg in 3.5 µl) promoted survival of RGCs to a greater extent, resulting in $191 \pm 13\%$, $205 \pm 14\%$ and $197 \pm 18\%$ surviving RGCs, respectively. A combination of both Y-27632 and CNTF equally resulted in a markedly increased survival of RGCs, without however surpassing the levels after CNTF application alone: $191 \pm 20\%$, $207 \pm 23\%$ and $192 \pm 26\%$ surviving RGCs compared to PBS-treated controls, respectively (Fig. 7B and C).

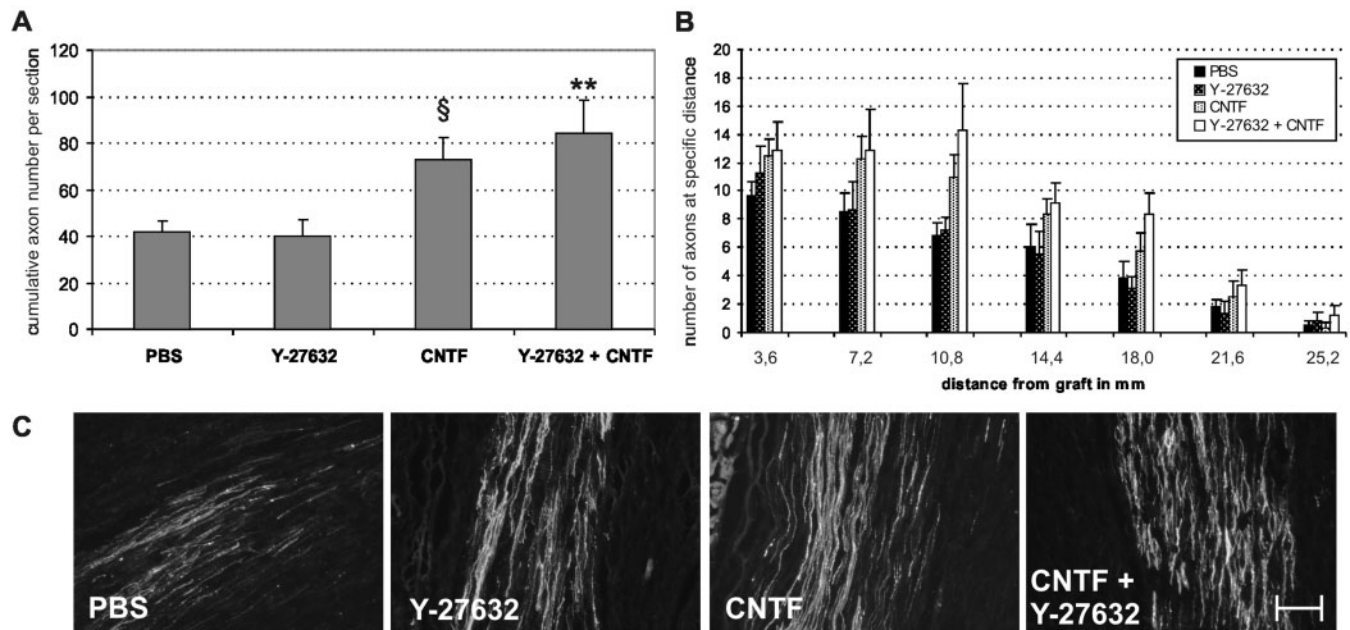


Fig. 8 Regeneration of retinal ganglion cell axons into a peripheral nerve graft after PBS-, CNTF-, Y-27632- and combination treatment. **(A)** Cumulative numbers of regenerating axons in a peripheral nerve graft section. Treatment with CNTF and the combination of CNTF and Y-27632 resulted in increased numbers of regenerating axons ($\$P = 0.06$; $**P = 0.003$ compared to PBS). **(B)** Outgrowth of regenerating axons beyond the graft site at specific distances. Animals treated with CNTF or the combination of CNTF and Y-27632 showed increased numbers of regenerating axons compared to PBS- and Y-27632-treated animals (n.s. for subgroup analysis) **(C)** Representative photomicrographs showing Cy3-CTB-stained regenerating axons of retinal ganglion cells in longitudinal sections of peripheral autografts at a distance of 10 mm from the graft site. Bar: 100 μ m.

Combinatorial treatment with CNTF and ROCK inhibitor improves RGC regeneration into the peripheral nerve graft

The sciatic nerve graft model allows the evaluation of RGC regeneration into a semi-permissive substrate. Since peripheral nerves lack major inhibitory molecules present in the CNS myelin, RGCs show a spontaneous regeneration response into the graft (Thanos *et al.*, 1997). The model thus permits to study a cumulative regenerative response reflecting the intrinsic regenerative capacity and the survival of RGCs. After transplantation of the sciatic nerve to the optic nerve stump, PBS, CNTF, Y-27632 and a combination of CNTF and Y-27632 were applied intravitreally and the application was repeated on Days 3 and 6 after surgery. Regenerating axons were labelled by intravitreal injection of Cy3-labelled cholera toxin subunit B 1 day before sacrificing the animals. In the control group (PBS-treated), the cumulative number of axons per section was 47.3 ± 9.2 , in the Y-27632-treated group 47.3 ± 12.4 (n.s. to PBS), in the CNTF-treated group 73.1 ± 16.7 ($P = 0.06$ to PBS) and in the group treated with a combination of Y-27632 and CNTF 87.5 ± 26 ($P = 0.003$ to PBS) (Fig. 8A and C). When the distances of axonal outgrowth from the graft site were compared, no differences were obvious between the PBS- and Y-27632-treated groups. Both CNTF- and combination-treated groups showed up to a 2-fold increase

in axon number up to a distance of 18 mm from the graft site. There was no difference between CNTF- and combination-treated groups (Fig. 8B).

Short-distance regeneration is potentiated by combinatorial CNTF- and ROCK-inhibitor treatment in the non-permissive optic nerve crush model

Finally, we used the optic nerve crush model to assess the regeneration response of RGC axons. In contrast to the peripheral nerve graft model, increased survival of axotomized RGCs alone is insufficient to overcome the non-permissive native CNS environment. Axonal outgrowth after optic nerve crush is thus used to quantify the potential of RGCs to overcome inhibitory myelin substrates.

Substance applications were performed immediately after the optic nerve crush and then subsequently at Days 3 and 6 after surgery. On Day 28 after crush animals were sacrificed and sections were immunostained for GAP-43 to identify regenerating axons. Control animals receiving PBS injections showed only minor regeneration past the lesion site. Application of CNTF significantly increased the number of regenerating axons resulting in up to ~ 2.5 -fold more regenerating axons compared to control. Intraocular injection of the ROCK-inhibitor Y-27632 increased the number of regenerating axons in a similar manner, resulting in ~ 2.5 -fold higher number of axons passing the

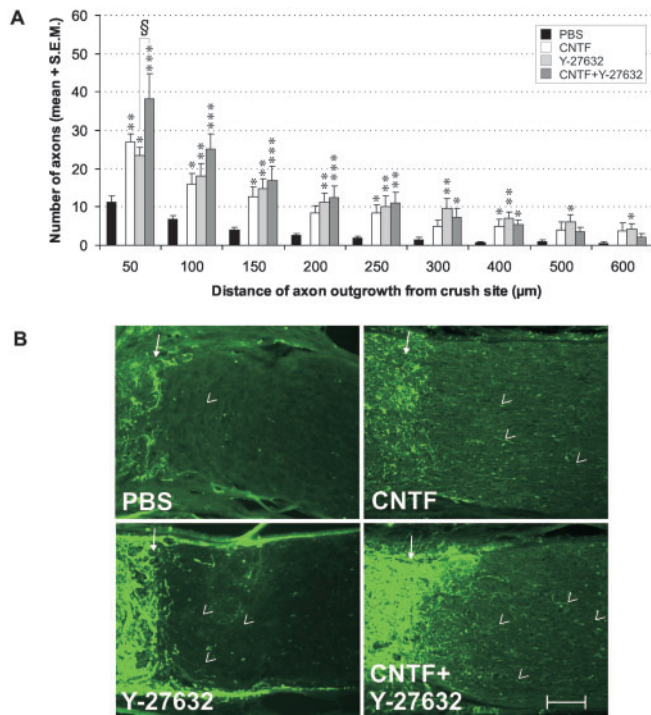


Fig. 9 Regeneration after optic nerve crush and application of CNTF and ROCK inhibitor. **(A)** Quantification of axonal regeneration past the crush site in animals treated with CNTF, Y-27632, a combination of both substances or PBS (Control). Significant increase in the number of regenerating axons after application of CNTF or Y-27632 (up to 2.5- to 3-fold compared to PBS controls). At smaller distances from the crush site the combination treatment was more potent than either substance alone (~1.6-fold compared to Y-27632-treated; $\$P < 0.05$). **(B)** Representative photomicrographs showing the crush site (arrow) and regenerating axons by GAP-43 immunofluorescence (arrow heads). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Bar: 100 μm .

crush site. Animals which received the combination treatment showed more regenerating axons past the crush site compared to the PBS group. In addition, the combination treatment was more effective than in the CNTF- or Y-27632-alone treatment group up to a distance of 250 μm past the crush site. At larger distances from the crush site, the combination treatment did not show any additional beneficial effect on axonal regeneration (Fig. 9).

Discussion

Regeneration in the CNS is limited by low intrinsic regenerative capacity of adult neurons, their subsequent apoptotic demise following lesion and by exogenous factors present in the neural tissue surrounding the lesioned axon. In this study, we have chosen a dual approach to simultaneously (i) counteract lesion-induced neuronal apoptosis, increase the regenerative capacity of lesioned RGCs via activation of intrinsic survival pathways; and (ii) attenuate growth inhibitory signalling cascades via interaction with a major effector molecule, ROCK.

We further set out to evaluate putative interactions of both cascades.

Inhibition of ROCK protects RGCs from serum- or growth factor-deprivation and axotomy-induced apoptosis

The Rho/ROCK pathway has previously been mainly associated with inhibitory signalling for neurite elongation (e.g. Bertrand *et al.*, 2005). Only little is known, however, about the role of this pathway in the regulation of cell survival. The inactivation of Rho, one of the upstream regulators of ROCK, has been shown to attenuate apoptosis following spinal cord injury which is dependent on p75^{NTR} (Dubreuil *et al.*, 2003). Neuronal survival was also shown to be enhanced by adeno-associated virus-mediated C3 expression, which however failed to show synergistic effects after additional lens injury (Fischer *et al.*, 2004). It remains unclear however, which cascades mediate cell survival downstream of Rho inactivation. In the present study, we show that ROCK inhibition protects RGCs from serum- and neurotrophin-deprivation (Figs 1 and 2). In the RGC-5 culture, the survival promoting effect of Y-27632 is comparable to saturating concentrations of CNTF and results in more pronounced effects when both substances are combined. ROCK inhibition also rescued RGCs from axotomy-induced apoptosis *in vivo* (Fig. 7B). To our knowledge this is the first report demonstrating neuroprotective effects of Y-27632, suggesting that ROCK activity may be detrimental for neuronal survival.

Differential regulation of intrinsic signalling cascades by Y-27632 and CNTF

The analysis of cell survival pathways revealed that ROCK inhibition resulted in MAPK phosphorylation thus activating one of the intracellular signalling cascades which is also employed by CNTF (Peterson *et al.*, 2000). The combination of both CNTF and Y-27632 resulted in an additive effect with an even more pronounced increase of MAPK phosphorylation (Fig. 5A and D). When MAPK was inhibited by PD 98059, RGC-5 cells grew shorter neurites in both CNTF- and Y-27632-treated groups, suggesting a major role for MAPK in neurite outgrowth (Fig. 6).

Levels of pSTAT3 were not markedly altered by application of Y-27632 alone (Fig. 5A and B). However, when cultures were treated with a combination of Y-27632 and CNTF, phosphorylation of STAT3 was markedly reduced compared to CNTF-alone treated cultures suggesting that ROCK inhibition results in the attenuation of the STAT3 response. Interestingly, inhibition of STAT3 by AG 490 further increased neurite outgrowth in RGC-5 cells, and this was most pronounced in both Y-27632 treated groups. STAT3 thus acts as a negative regulator of neurite outgrowth and the inhibition of STAT3 phosphorylation by Y-27632 further increases neurite elongation. A link

between RhoA and STAT3 signalling has been previously established in mouse fibroblasts, where RhoA has been shown to signal via Rho kinase and STAT3 (Debidda *et al.*, 2005).

Combination treatment by Y-27632 and CNTF also exerted additive effect on p-Akt levels (Fig. 5A and F). Inhibition of Akt by Triciribine resulted in shorter neurites and increased cell death in RGC-5 cells (Fig. 6). Akt thus promotes neurite elongation and cell survival and this pathway is equally employed by Y-27632 and CNTF.

Taken together, inhibition of ROCK in our paradigm attenuates some of the CNTF-mediated effects (via STAT3) while other pathways are triggered in an additive manner (MAPK and Akt). Based on the fact that STAT3 activation is a major promoter of RGC survival in several models of axonal lesion, the cross-talk of Y-27632 with STAT3 phosphorylation may be responsible for the lack of an additive effect on RGC survival in the combination treatment group, as observed in our axotomy model *in vivo* (Fig. 7B and C). On the other hand, inhibition of STAT3 signalling increases the effects of Y-27632 on neurite elongation. In addition to specific effects of Y-27632 on ROCK, non-specific effects of Y-27632 on other kinases have also to be considered: we have previously shown that among several tested kinase activities the K_i values of Y-27632 were lowest for ROCK-II, but activities of PKN and PKC delta were also inhibited to a lesser extent (Lingor *et al.*, 2007). Since PKC delta has been implicated in ERK 1/2 activation (Choi *et al.*, 2006), its inhibition may interfere with survival mediating signals.

Vice versa, the application of Y-27632 and CNTF was diminishing ROCK activity in culture more than either substance alone (Fig. 5L). Thus, in addition to the effects on survival, the combination of CNTF and Y-27632 is able to modulate the growth inhibitory pathway in an additive manner.

Upregulation of ROCK is a specific axotomy-induced response in RGC

Mechanical lesions in the CNS, such as crush or axotomy, result in an exposure of damaged axons to inhibitory substrates. We thus asked, whether axotomy may lead to an upregulation of mediators of inhibitory signalling, such as ROCK. In our study, immunohistochemical analysis of retinae at different time points after axotomy revealed an upregulation of ROCK-II with a maximum at Day 4 following optic nerve lesion (Fig. 7A). ROCK-II upregulation was not observed in other retinal layers, implicating that this is a specific response of RGCs to axonal lesion. In hypoglossal nerves, upstream activators of ROCK, like RhoA, and other Rho family GTPases, like Rac1, Cdc42 and TC10, have equally been shown to be upregulated after axotomy (Tanabe *et al.*, 2000). This adds members of the Rho/ROCK pathway to the list of lesion-induced molecules in CNS neurons, such as the immediate-early genes c-Jun

or c-Fos (Hull and Bahr, 1994). Upregulation of ROCK-II *per se* does not implicate its activation, but it suggests a greater availability of this protein for a subsequent activation step, which is similar for the upregulation of c-Jun after optic nerve axotomy (Hull and Bahr, 1994). Downregulation of c-Jun by siRNA-injection into the optic nerve stump was therefore able to rescue RGCs from axotomy-induced apoptosis (Lingor *et al.*, 2005). Similarly, increased expression of ROCK-II following nerve lesion may argue for its participation in regeneration-inhibitory cascades, but it may also reflect a compensatory mechanism in an inefficient regenerative attempt. We therefore asked the question whether lesion-induced upregulation of ROCK-II may play a detrimental role for RGC regeneration and whether inhibition of ROCK activity by Y-27632 had beneficial effects for survival and regeneration *in vivo*.

RGC regeneration *in vivo* is enhanced by a Y-27632/CNTF combination treatment

The peripheral nerve graft model is a semi-permissive model for regeneration: although peripheral nerves do not express Nogo-A (Pot *et al.*, 2002), they show expression of other inhibitory molecules, e.g. Sema-III or MAG, similar to those found in the CNS (Pasterkamp *et al.*, 1998; Gupta *et al.*, 2006). Unlike into the native optic nerve, regenerating axons regrow into a peripheral nerve graft even at control conditions (Fig. 8). It is thus not surprising, that inhibition of ROCK by Y-27632 alone did not significantly alter the regeneration response in this model. However, co-application of Y-27632 and CNTF increased the number of regenerating axons into the peripheral nerve graft with a trend to be more potent than CNTF alone, suggesting a beneficial interaction of CNTF and Y-27632 even in the semi-permissive environment (Fig. 8).

The effect on regeneration was more pronounced in the optic nerve crush model where animals treated with a combination of Y-27632 and CNTF showed a stronger regeneration at distances up to 250 μ m from the crush site compared to CNTF- or Y-27632-alone treatments (Fig. 9). Both the STAT3 and the MAPK pathway have been recently suggested to affect regeneration of RGCs (Kretz *et al.*, 2005; Rios-Munoz *et al.*, 2005). Our results suggest that axonal regeneration of RGC *in vivo* depends less on STAT3 and more on MAPK signalling, since the co-application of Y-27632 and CNTF still resulted in beneficial effects. This is supported by the *in vitro* data using specific inhibitors for STAT3 (AG 490) and MAPK (PD 98059) (Fig. 6).

In general, protective and pro-regenerative effects of the combination treatment appear to be more pronounced *in vitro* than *in vivo* in this study. While we used RGC-5 cells and primary RGCs of P7-8 pups for *in vitro* experiments, all *in vivo* studies were performed on adult rats. In comparison to embryonic and postnatal RGCs the regenerative potential of adult RGCs is markedly lower (Chierzi *et al.*, 2005; Verma *et al.*, 2005). Our results thus

additionally support the concept that in the adult CNS, the response to pro-regenerative cues, in our case CNTF and Y-27632, is decreased.

In summary, our data shows beneficial effects of ROCK inhibition by Y-27632 and CNTF on both survival and neurite outgrowth/regeneration with additive effects on both parameters compared to each substance alone. Interestingly, ROCK inhibition by Y-27632 results in MAPK and Akt phosphorylation, thus employing a signalling cascade commonly triggered by neurotrophic factors for the mediation of cell survival. We suggest that the combinatorial effects are mediated via synergistic activation of the MAPK and Akt pathways, while inhibition of STAT3 phosphorylation by Y-27632 may account for a partial attenuation of a beneficial effect on cell survival. ROCK clearly represents an independent pharmacological target next to RhoA: besides being activated by RhoA, other molecules have been implicated to directly activate ROCK (Fu *et al.*, 1998; Shirao *et al.*, 2002) and RhoA on the other hand may activate molecules different from ROCK (Ridley, 2006). From the translational point of view, the regulation of ROCK by small molecule pharmacological inhibitors bears another major advantage: in contrast to RhoA inhibitors, ROCK inhibitors are already at present in clinical use facilitating their putative therapeutic application for CNS injury (Wettschureck and Offermanns, 2002; Lai and Frishman, 2005). Although a combinatorial approach targeting apoptosis and inhibitory pathways holds promise for an increased functional regeneration, a cross-talk of intracellular pathways may differentially enhance and at the same time limit beneficial effects, which has to be taken into account in the development of further therapeutic strategies.

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