NEURAL REGENERATION RESEARCH
December 2015, Volume 10, Issue 12
www.nrronline.org

PERSPECTIVE

ROCK inhibition as a novel potential strategy for axonal regeneration in optic neuropathies

Optic neuropathies or optic nerve diseases are a frequent cause of permanent vision loss that can occur after inflammation, ischemia, infection, tumors, trauma and/or an elevated pressure inside the eye (also called intraocular pressure or IOP). Glaucoma or glaucomatous optic neuropathy is the most commonly acquired optic neuropathy and the second leading cause of blindness worldwide. This neurodegenerative disorder is characterized by a slow and progressive loss of retinal ganglion cells (RGCs) and their axons, and is often associated with an elevated IOP. Current glaucoma treatments therefore focus on reducing the raised IOP. Unfortunately, not all patients benefit from an IOP-lowering therapy, also because the pathophysiology of this multifactorial disease is not merely associated with an altered eye pressure. The exact mechanisms underlying apoptotic RGC death, which is a common feature of all types of glaucoma, remains complex and largely unsolved. Therefore, new therapeutic strategies should focus on preventing or retarding RGC death, but also on sufficient and lengthy repair/regrowth of damaged RGC axons in the optic nerve and on proper axonal guidance, all in order to preserve or improve structural and functional connectivity and ultimately restore vision. Unfortunately, the damaged or diseased mammalian central nervous system (CNS) is characterized by poor axonal regeneration, which is generally believed to depend on a combination of factors, in particular the presence of reactive astrocytes, oligodendrocytes, and their associated inhibitory molecules, and the insufficient intrinsic growth capacity of mature CNS neurons. Yet, also the loss of neurotrophic support, apoptotic cell death and poor debris clearance contribute to this regenerative failure. To date, no clinical therapy is available to cure the damaged CNS, although there has been considerable progress in understanding the underlying mechanisms of regenerative failure and in providing possible ways to achieve long-distance regeneration. In order to develop novel potential regenerative strategies and treatments, the optic nerve crush (ONC) paradigm has been a frequently used *in vivo* rodent model over the past decades. Indeed, research using this model resulted in novel insights into the destructive cellular and molecular pathways underlying axonal degeneration and RGC death, and importantly contributed to the discovery of potential axon growth and guidance-stimulating molecules and treatments (Van de Velde et al., 2015). Within our research group, ONC injury in mice has been frequently used as an experimental model to mimic glaucoma pathology, in order to identify novel neuroprotective/regenerative molecules, such as Rho kinase inhibitors. Within this perspective, we aim to highlight the current status of research on Rho-associated coiled-coil protein kinase (ROCK) in the promotion of neurite outgrowth and axonal regeneration in experimental optic neuropathy models.

Over the past years, the selective inhibition of ROCK was shown as an efficient strategy to support axonal outgrowth and regeneration in animal models of CNS trauma/disease. This intracellular serine/threonine protein kinase acts downstream of the GTPase Rho and consists in two isoforms, ROCKI and II. ROCK phosphorylates various substrates, such as myosin light chain (MLC) phosphatase, LIM kinase, glial fibrillary acidic protein (GFAP), collapsin response mediator proteins (CRMPs), and regulates several cellular functions, such as actin-cytoskeleton assembly, formation of stress fibers, cell migration, phagocytosis and apoptosis, thereby contributing to a multitude of (patho)physiological processes. In addition, the Rho/ROCK pathway has been suggested to negatively modulate neurite extension during development and axon regeneration, resulting in axon retraction and growth cone collapse (Van de Velde et al., 2015). Notably, many of the known/suggested upstream and downstream signaling molecules of the ROCK pathway have been implicated in the restricted axonal regeneration potential of the adult mammalian CNS, *e.g.*, CRMP-2, phosphatase and tensin homolog (PTEN), myelin-associated inhibitors (Nogo, myelin-associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMgp)), glial-derived inhibitory ligands such as chondroitin sulfate proteoglycans (CSPGs), *etc.* (Van de Velde et al., 2015). Thus, its presumed broad field of action makes blocking the Rho-ROCK pathway a promising strategy to promote axonal regeneration and repair in the damaged CNS.

A detailed description of all *in vitro*, *ex vivo* and *in vivo* studies describing ROCK inhibition/knockdown as a potential strategy to achieve neuroprotection and/or axonal regeneration in the visual system, has recently been reviewed by our group (Van de Velde et al., 2015). Most studies used the ROCK inhibitor Y-27632, which belongs, together with the more potent Y-39983, to the 4-aminopyridin derivates, and should be more specific as compared to the widely used ROCK inhibitor, fasudil, an isoquinoline derivate. *In vitro* studies using postnatal rat primary RGCs, grown on growth-permissive substrates, showed induced neurite outgrowth and lengthening after addition of Y-27632, Y-33983 or after specific viral vector-mediated ROCKII knockdown (AAV.ROCKII-shRNA). These findings could also be observed in non-permissive circumstances, where postnatal RGCs were grown on CSPGs and/or Nogo-A, MAG and OMgp, suggesting that ROCK lies downstream of these inhibitory molecules and that suppressing ROCK might relief the repulsive effects of glial/myelin-derived inhibitory ligands (Lingor et al., 2008; Bermel et al., 2009; Tokushige et al., 2011; Koch et al., 2014). Moreover, combining ROCK inhibition with administration of the growth factor ciliary neurotrophic factor (CNTF) resulted in an increased amount and length of outgrowing neurites (Lingor et al., 2008). Also on CNS myelin-inhibited adult retinal cells, which lack the growth-promoting effect of cAMP, the combination of CNTF and Y-27632 obviously enhanced neurite outgrowth, in contrast to single application of Y-27632, which did not affect neurite outgrowth in these cells (Ahmed et al., 2009). In addition, the combined administration of Y-27632, CNTF and forskolin, which raises intracellular cAMP levels, on adult RGCs resulted in a stronger boost of neurite outgrowth than Y-27632 and/or forskolin treatment alone (Ahmed et al., 2009). These findings already indicate the axon growth-promoting potential of ROCK inhibitors, as well as their promising role in obtaining synergistic effects in combination treatments.

Next to the cellular assays, retinal explant cultures have been used to further explore the effects of ROCK inhibition on neurite outgrowth. These *ex vivo* cultures form an ideal model system as they are easily manageable and more closely resemble the *in vivo* situation as compared to cell cultures. When using embryonic chicken retinal explants, cultured in the presence of CSPG substrates, administration of Y-27632 reduced the CSPG-mediated inhibition on neurite outgrowth/length, indicating that CSPG-induced inhibition of growth cones is partially mediated by the ROCK signaling pathway (Van de Velde et al., 2015). Interestingly, using a postnatal mouse retinal explant model, our group clearly showed a neurite outgrowth-promoting effect of both Y-39983 and Y-27632, with Y-39983 being more potent in stimulating the initiation of outgrowing neurites as compared to Y-27632 (Van de Velde et al., 2015). Our findings correspond to the reported effects of Y-39983 and Y-27632 on neurite outgrowth in adult cat retinal explants (Sagawa et al., 2007). Strikingly, in contrast to brain-derived neurotrophic factor (BDNF)/CNTF administration, which highly stimulates extension of neurites, Y-39983 enhanced initiation of neurite outgrowth rather than neurite elongation (**Figure 1**) (Van Hove et al., unpublished data). Yet, also supplementation of Y-27632 or Y-39983 on adult cat/rat retinal explants resulted in increased neurite outgrowth initiation, rather than elongation (Sagawa et al., 2007; Ichikawa et al., 2008; Bermel et al., 2009), which was even more enhanced when applied together with a cyclin dependent kinase 5 (cdk5) inhibitor (Bermel et al., 2009). All together, these *in vitro*/*ex vivo* findings indicate that inhibition of ROCK has a strong potential to support axonal outgrowth, even in the presence of growth-inhibitory ligands. The data also suggest that combining ROCK inhibition, which seems to support neurite outgrowth initiation, with neurite elongation stimulating compounds might be a good strategy to induce robust axonal outgrowth/regeneration.

To confirm these *in vitro* and *ex vivo* findings, several *in vivo* studies were performed. Intravitreal injections of Y-27632, Y-39983 or AAV.ROCKII-shRNA after ONC in the rat or cat augmented the number and elongation of regenerating axons (Lingor et al., 2007, 2008; Sagawa et al., 2007; Ichikawa et al., 2008; Koch et al., 2014). Yet, fasudil and dimethylfasudil were not able to induce axonal outgrowth, neither in the rat or cat ONC model (Lingor et al., 2007; Ichikawa et al., 2008),

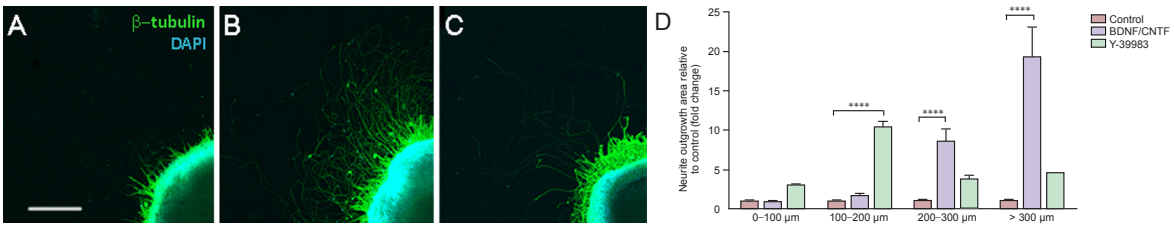

Figure 1 Neurite outgrowth of ROCK inhibitor-treated postnatal mouse retinal explants.
Representative pictures of (A) untreated explants and (B) explants treated with BDNF (5 ng/mL)/CNTF (1 ng/mL) and (C) Y-39983 (5 µM), stained for β-tubulin (green), labeling neuronal processes, and DAPI (blue), labeling the cells in the explant body, after 3 days in culture. (D) Neurite outgrowth was investigated by categorizing the outgrowing neurites into 4 segments, developed by successive 100 µm increments in radius from the explant body, which results in 3 ring segments and an outer segment covering the remaining immunolabeled neurites. Treatment of explants with BDNF/CNTF clearly resulted in a significant increase in neurite elongation, observable by the high amount of axons in the outer segment, as compared with explants treated with Y-39983, which revealed induced neurite outgrowth initiation, as areas close to the explant bodies contained relatively more axons. Data are represented as mean ± SEM; n ≥ 18; ****$P < 0.0001$. Scale bar: 200 µm; BDNF: Brain-derived neurotrophic factor; CNTF: ciliary neurotrophic factor.

indicating that more specific and potent ROCK inhibitors are required to obtain growth stimulation. Similarly, Y-39983, but not Y-27632, promoted axonal regeneration in a rat peripheral nerve graft model, in which the sciatic nerve is transplanted onto the optic nerve stump (Lingor et al., 2008; Tokushige et al., 2011). Also application of Y-27632 in a mouse ONC model did not result in a significantly induced regenerative response (Pernet et al., 2013). However, combined treatment of Y-27632 with other outgrowth-stimulating compounds such as a Cdk5 inhibitor or CNTF, in the ONC model and/or peripheral nerve graft model, boosted regeneration and resulted in an increased number, rather than length of the regenerating axons compared to application of each compound alone, again indicative for advantageous interplays between these molecules. Interestingly, intraocular delivery of Y-27632 combined with AAV2.STAT3-ca in RGCs or ShH10.DH-CNTF in Müller glia, increased the number and length of growing axons even more, promoted axons to grow in straighter paths and reduced the amount of U-turns, as compared to single AAV2.STAT3-ca or ShH10.DH-CNTF application (Pernet et al., 2013). These findings demonstrate that inhibition of ROCK counteracted the Rho-mediated misguidance effects induced by, amongst others, myelin-associated inhibitory ligands, thereby resulting in a more growth-permissive local environment.

As previously mentioned, maintenance or recovery of functional connectivity requires, next to (long-distance) axonal regeneration of surviving neurons and proper guidance towards the appropriate target area, also survival of axotomized neurons and prevention of secondary degeneration. Once the devastating Rho-ROCK cascade is activated after an acute traumatic injury or in chronic neurodegenerative diseases, axonal outgrowth, but also cell survival becomes compromised. While a multitude of reported studies unveiled a potential role for ROCK inhibition in axonal regeneration, a limited number already also clearly elucidated ROCK inhibition as a novel neuroprotective therapy in optic neuropathy models (Lingor et al., 2008; Bermel et al., 2009; Koch et al., 2014; Van de Velde et al., 2015). Furthermore, reduced neurodegeneration after ROCK inhibition has also been demonstrated in experimental (animal) models for e.g., Alzheimer's and Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis and stroke (Van de Velde et al., 2015).

Overall, ROCK signaling is clearly involved in a multitude of pathways, which are still mostly undiscovered in the injured/diseased CNS, thereby contributing to many pathological features, which prompts this kinase as a central target for the treatment of neurodegenerative disorders, such as glaucoma. It is increasingly recognized that strategies that aim to repair the functional connections following injury/lesions should attempt to target multiple pathways. ROCK inhibition or ROCK knockdown strategies clearly enables the stimulation of several repair processes and seems therefore, albeit in combination with other (growth)factors, a potential therapy for the treatment of this degenerative disease. Hence, it remains important to profoundly understand the pathological pathways and mechanisms underlying neurodegeneration and the restricted regeneration as it exists in the adult mammalian CNS. Interestingly, recent advances in the field have resulted in the identification and characterization of multiple novel candidate molecules/treatments able to support or induce processes related to neuroprotection and/or regeneration. Novel studies should consider these recent discoveries to create the best complementary combinatorial approach focusing on *e.g.*, intrinsic growth stimulation with neutralization of glia/myelin-associated growth inhibitory factors, in order to obtain sufficient and proper regenerating axons in the brain target areas, thereby ultimately restoring functional connections. ROCK inhibition might as such be a versatile strategic partner in the search for novel treatment strategies for glaucoma, yet also for other neurodegenerative disorders.

The authors are financially supported by the Hercules Grant [AKUL/09/038] and national Grants from the Research Council of KU Leuven [KU Leuven BOF-OT/10/033] and the Flemish Institute for the promotion of scientific research (IWT and FWO).

Inge Van Hove, Evy Lefevere, Lieve Moons *

Neural Circuit Development and Regeneration Research Group, Department of Biology, KU Leuven, Leuven, Belgium (Van Hove I, Lefevere E, Moons L)
Laboratory of Ophthalmology, Department of Neurosciences, KU Leuven, Leuven, Belgium (Van Hove I)

***Correspondence to:** *Lieve Moons, Ph.D., Lieve.moons@bio.kuleuven.be.*
***Accepted:** 2015-11-13*
***orcid:** 0000-0002-3125-0438 (Inge Van Hove)*
0000-0001-8145-1070 (Evy Lefevere)
0000-0003-0186-1411 (Lieve Moons)
***doi:** 10.4103/1673-5374.172311* ***http**://www.nrronline.org/*
Van Hove I, Lefevere E, Moons L (2015) ROCK inhibition as a novel potential strategy for axonal regeneration in optic neuropathies. Neural Regen Res 10(12):1949-1950.

References

Ahmed Z, Berry M, Logan A (2009) ROCK inhibition promotes adult retinal ganglion cell neurite outgrowth only in the presence of growth promoting factors. Mol Cell Neurosci 42:128-133.
Bermel C, Tonges L, Planchamp V, Gillardon F, Weishaupt JH, Dietz GP, Bahr M, Lingor P (2009) Combined inhibition of Cdk5 and ROCK additively increase cell survival, but not the regenerative response in regenerating retinal ganglion cells. Mol Cell Neurosci 42:427-437.
Ichikawa M, Yoshida J, Saito K, Sagawa H, Tokita Y, Watanabe M (2008) Differential effects of two ROCK inhibitors, Fasudil and Y-27632, on optic nerve regeneration in adult cats. Brain Res 1201:23-33.
Koch JC, Tonges L, Barski E, Michel U, Bahr M, Lingor P (2014) ROCK2 is a major regulator of axonal degeneration, neuronal death and axonal regeneration in the CNS. Cell Death Dis 5:e1225.
Lingor P, Teusch N, Schwarz K, Mueller R, Mack H, Bahr M, Mueller BK (2007) Inhibition of Rho kinase (ROCK) increases neurite outgrowth on chondroitin sulphate proteoglycan in vitro and axonal regeneration in the adult optic nerve in vivo. J Neurochem 103:181-189.
Lingor P, Tonges L, Pieper N, Bermel C, Barski E, Planchamp V, Bahr M (2008) ROCK inhibition and CNTF interact on intrinsic signalling pathways and differentially regulate survival and regeneration in retinal ganglion cells. Brain 131:250-263.
Pernet V, Joly S, Jordi N, Dalkara D, Guzik-Kornacka A, Flannery JG, Schwab ME (2013) Misguidance and modulation of axonal regeneration by Stat3 and Rho/ROCK signaling in the transparent optic nerve. Cell Death Dis 4:e734.
Sagawa H, Terasaki H, Nakamura M, Ichikawa M, Yata T, Tokita Y, Watanabe M (2007) A novel ROCK inhibitor, Y-39983, promotes regeneration of crushed axons of retinal ganglion cells into the optic nerve of adult cats. Exp Neurol 205:230-240.
Tokushige H, Waki M, Takayama Y, Tanihara H (2011) Effects of Y-39983, a selective Rho-associated protein kinase inhibitor, on blood flow in optic nerve head in rabbits and axonal regeneration of retinal ganglion cells in rats. Curr Eye Res 36:964-970.
Van de Velde S, De Groef L, Stalmans I, Moons L, Van Hove I (2015) Towards axonal regeneration and neuroprotection in glaucoma: Rho kinase inhibitors as promising therapeutics. Prog Neurobiol 131:105-119.