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AAV.shRNA-mediated downregulation of ROCK2 attenuates degeneration of dopaminergic neurons in toxin-induced models of Parkinson's disease in vitro and in vivo

Kim-Ann Saal [a,1], Jan C. Koch [a,1], Lars Tatenhorst [a], Éva M. Szegő [b], Vinicius Toledo Ribas [a], Uwe Michel [a], Mathias Bähr [a,c], Lars Tönges [a,c], Paul Lingor [a,c,*]

[a] Department of Neurology, University Medicine Göttingen, Robert-Koch-Str. 40, 37075 Göttingen, Germany
[b] Department of Neurodegeneration and Restorative Research, University of Göttingen, Waldweg 33, 37073 Göttingen, Germany
[c] Center Nanoscale Microscopy and Molecular Physiology of the Brain (CNMPB), Göttingen, Germany

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder with prominent neuronal cell death in the substantia nigra (SN) and other parts of the brain. Previous studies in models of traumatic and neurodegenerative CNS disease showed that pharmacological inhibition of Rho-associated kinase (ROCK), a molecule involved in inhibitory signaling in the CNS, by small-molecule inhibitors improves neuronal survival and increases regeneration. Most small-molecule inhibitors, however, offer only limited target specificity and also inhibit other kinases, including both ROCK isoforms. To establish the role of the predominantly brain-expressed ROCK2 isoform in models of regeneration and PD, we used adeno-associated viral vectors (AAV) to specifically knockdown ROCK2 in neurons. Rat primary midbrain neurons (PMN) were transduced with AAV expressing short-hairpin-RNA (shRNA) against ROCK2 and LIM-domain kinase 1 (LIMK1), one of the downstream targets of ROCK2. While knock-down of ROCK2 and LIMK1 both enhanced neurite regeneration in a traumatic scratch lesion model, only ROCK2-shRNA protected PMN against 1-methyl-4-phenylpyridinium (MPP^+) toxicity. Moreover, AAV.ROCK2-shRNA increased levels of the pro-survival markers Bcl-2 and phospho-Erk1.

In vivo, AAV.ROCK2-shRNA vectors were injected into the ipsilateral SN and a unilateral 6-OHDA striatal lesion was performed. After four weeks, behavioral, immunohistochemical and biochemical alterations were investigated. Downregulation of ROCK2 protected dopaminergic neurons in the SN from 6-OHDA-induced degeneration and resulted in significantly increased TH-positive neuron numbers. This effect, however, was confined to nigral neuronal somata as striatal terminal density, dopamine and metabolite levels were not significantly preserved. Interestingly, motor behavior was improved in the ROCK2-shRNA treated animals compared to control after four weeks.

Our studies thus confirm ROCK2 as a promising therapeutic target in models of PD and demonstrate that neuron-specific inhibition of ROCK2 promotes survival of lesioned dopaminergic neurons.

Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder characterized by progressive neuronal cell death in the substantia nigra (SN) and other regions of the brain. Although motor symptoms of PD, e.g. bradykinesia, rigor and tremor, can be ameliorated by numerous symptomatic treatments, there is still no etiological therapy available. Disease-modifying treatments should address not only neuronal cell death but also axonal degeneration, which represents one of the first steps in the pathophysiology of PD (Burke and O'Malley, 2013).

Rho-associated kinase (ROCK) is involved in the intracellular propagation of extracellular cues signaling axonal growth arrest and regenerative block. Amongst other upstream regulators, ROCK can be activated by RhoA after binding of extracellular inhibitory substrates, e.g. Nogo,

Abbreviations: 6-OHDA, 6-hydroxydopamine; AAV, adeno-associated viral vector; Bcl-2, B-cell lymphoma 2; CNS, central nervous system; ctl, contralateral; DAPI, 4′,6-diamidino-2-phenylindole; DIV, day in vitro; DOPAC, 3,4-dihydroxyphenylacetic acid; EGFP, enhanced green fluorescent protein; Erk, extracellular-signal-regulated kinase; GFAP, glial fibrillary acidic protein; HPLC, high performance liquid chromatography; HVA, homovanillic acid; Iba1, ionized calcium binding adapter molecule 1; ipl, ipsilateral; LIMK, LIM domain kinase; MAPK, mitogen-activated protein kinase; PMN, primary midbrain neurons; MPP^+, 1-methyl-4-phenylpyridinium; PD, Parkinson's disease; PTEN, phosphatase and tensin homolog deleted on chromosome ten; ROCK, Rho-associated protein kinase; shRNA, short hairpin ribonucleic acid; SN, substantia nigra; Stat3, signal transducer and activator of transcription 3; TH, tyrosine hydroxylase; TU, transforming units.

* Corresponding author at: Department of Neurology, University Medicine Göttingen, Robert-Koch-Str. 40, D-37075 Göttingen, Germany. Fax: +49 551 39 8405.
E-mail address: plingor@gwdg.de (P. Lingor).
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[1] These authors contributed equally to this work.

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oligodendrocyte myelin glycoprotein (OMgp) or myelin associated glycoprotein (MAG), to their receptors (Geoffroy and Zheng, 2014). One of the main downstream targets of ROCK is LIM domain kinase (LIMK), which is known to inactivate the actin-depolymerizing enzyme cofilin resulting in reduced actin turnover and growth cone collapse (Endo et al., 2003). More recently, ROCK was also shown to regulate cellular pathways which mediate neuronal survival and axonal degeneration (Koch et al., 2014; Tönges et al., 2011a; Tura et al., 2009; Yamashita et al., 2007). ROCK exists in two isoforms with high homology, ROCK1 (or ROKβ) and ROCK2 (or ROKα). Whereas ROCK1 is mostly expressed in non-neuronal tissue, ROCK2 expression is strong in the CNS and spinal cord (Duffy et al., 2009; Hashimoto et al., 1999). These properties underline the role of ROCK2 as a highly interesting molecular target for the treatment of neurodegenerative disorders (Tönges et al., 2011a).

In previous studies in models of traumatic and neurodegenerative CNS disease others as well as our group could show that pharmacologic ROCK inhibition attenuates neurodegeneration and improves axonal regeneration (Lingor et al., 2007, 2008; Rodriguez-Perez et al., 2013; Tönges et al., 2014; Villar-Cheda et al., 2012). The neuroprotective effects of pharmacologic ROCK inhibition have been ascribed not only to its direct action on neurons (Tönges et al., 2012; Yamashita et al., 2007), but also on astrocytes (Lau et al., 2012), microglia (Barcia et al., 2012; Borrajo et al., 2014; Ding et al., 2010; Tönges et al., 2014; Villar-Cheda et al., 2012; Zhang et al., 2013) and leukocytes (Hirata et al., 2008; Sun et al., 2006). We have recently tested the effects of the pharmacological ROCK-inhibitor fasudil in two in vivo animal models of PD: In the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin (MPTP) model, fasudil treatment resulted in a significant attenuation of dopaminergic cell loss, preservation of dopaminergic terminals and improvement in motor performance (Tönges et al., 2012). In the more severe 6-hydroxydopamine (6-OHDA) mouse model of PD on the other hand, nigral dopaminergic cell survival was not significantly improved after high dose fasudil treatment. However, HPLC analysis of dopamine metabolites revealed significantly increased striatal levels of 3,4-dihydroxyphenylacetic acid (DOPAC) in the fasudil-treated animals at 12 weeks after 6-OHDA lesion, suggesting a regenerative response (Tatenhorst et al., 2014).

One drawback of the frequently used pharmacologic kinase inhibitors, such as the isochinoline derivative fasudil and the 4-aminopyridine Y-27632, is their incomplete specificity towards the target, which implies a more or less pronounced regulation of other kinases (Davies et al., 2000). Off-target effects may thus mask the effects on the original kinase and may be derived from the regulation of other molecular pathways. For the design of targeted therapies, however, the precise characterization of the pharmacologic target is urgently required.

In this study we therefore aimed to investigate the neuron-specific impact of ROCK2 inhibition in toxin-induced models of neurodegeneration in vitro and in vivo. By using a highly selective viral shRNA-mediated gene silencing approach targeting brain specific ROCK2 in a severe 6-OHDA mouse model of PD in vivo as well as ROCK2 and its downstream target LIMK1 in a MPP^+ toxin-induced model of primary midbrain neurons (PMN) in vitro, we investigated the neuron-specific downregulation of ROCK2 to clarify its impact in the cell type most affected in PD.

Material and methods

Cloning and production of AAV

We produced adeno-associated viral vectors (AAV) co-expressing a short hairpin RNA (shRNA) under control of the H1-promoter and the fluorophore EGFP or dsRed under control of the neuron-specific human synapsin promoter. For cloning of the AAV-plasmids, the oligonucleotides of previously tested siRNAs against ROCK2, LIMK1 and firefly as control were cloned in the vector pSUPER-hSyn-EGFP-CytB-AS (GenBank ID: AY640629), respectively, as described before (Koch et al., 2014; Michel et al., 2005).

The sequences of the shRNA primers were as follows (in bold: sequence of the respective siRNA-sense and -antisense strand, in italics: sequence of the hairpin turn):

ROCK2-shRNA forward primer:5′-GATCCCC**TGCAAAGTTTATTATGATATA***CTTCCTGTCA***TATATCATAATAAACTTTGCA**TTTTTGGAAA-3′.

ROCK2-shRNA reverse primer:5′-AGCTTTTCCAAAAA**TGCAAAGTTTATTATGATATA***TGACAGGAAG***TATATCATAATAAACTTTGCA**GGG-3′.

LIMK1-shRNA forward primer:5′-GATCCCC**AACACCTAGGGACGTATTAAA***CTTCCTGTCA***TTTAATACGTCCCTAGGTGTT**TTTTTGGAAA-3′.

LIMK1-shRNA reverse primer:5′-AGCTTTTCCAAAAA**AACACCTAGGGACGTATTAAA***TGACAGGAAG***TTTAATACGTCCCTAGGTGTT**GGG-3′.

firefly-shRNA forward primer:5′-GATCCCC**CTGACGCGGAATACTTCGA***TTCAAGAGA***TCGAAGTATTCCGCGTCAG**TTTTTGGAAA-3′

firefly-shRNA reverse primer:5′-AGCTTTTCCAAAAA**CTGACGCGGAATACTTCGA***TCTCTTGAA***TCGAAGTATTCCGCGTCAG**GGG-3′

The resulting vectors were digested with BstB1/Hind3 and the fragments containing the H1-promoter and the shRNA were cloned into a BstB1/Hind3-cut pAAV-9(5)hSyn-EGFP-CytbAS-ohneNot (GenBank ID: HQ416702), resulting in the vectors pAAV-9(5)hSyn-EGFP-H1-ROCK2-shRNA, -LIMK1-shRNA and firefly-shRNA. Cloning of the corresponding vector pAAV-9(5)hSyn-DsRed-H1-ROCK2-shRNA, expressing ROCK2-shRNA and the fluorophore dsRed, and the respective control vector pAAV-9(5)hSyn-DsRed-H1-EGFP-shRNA (= pAAV-Si-I (GenBank ID: AY6406334)), expressing an shRNA against EGFP and the fluorophore dsRed, have been described before (Koch et al., 2014). All plasmids were sequenced to confirm their correct identity, and then used for production of AAV as reported before (Zolotukhin et al., 1999). In brief, 293-HEK cells were transfected with calcium phosphate, HEPES-buffered saline and a serotype-specific plasmid mix (for AAV2: pAAV-RC, pHELPER (both from Stratagene, La Jolla, CA, USA) and the respective pAAV expression vector in a 1: 1: 1 molar ratio; for AAV1/2: pAAV-RC, pH21, pHELPER and the respective pAAV expression vectors in a 0.5: 0.5: 1: 1 molar ratio). 48 h after transfection, cells were harvested and AAV were purified by dialysis and virus gradient centrifugation in iodixanol. To obtain high titer viral stocks, fast protein liquid chromatography (FPLC) was performed. The virus stocks were tested on primary cortical neurons for transduction efficacy and toxicity and viral titers were determined using qPCR. For all in vitro experiments we employed the hybrid serotype AAV1/2 consisting of the AAV2 inverted terminal repeats packed into AAV1/AAV2 hybrid capsids (molar ratio 1: 1) because this serotype leads to a more efficient and rapid transduction of neurons in vitro (Koch et al., 2013). For all in vivo experiments, the serotype AAV2 was used as it is best established for this paradigm as targeting primarily neurons (Löw and Aebischer, 2012; Tenenbaum L. et al., 2000).

PMN culture, immunocytochemistry, in vitro assays of dopaminergic cell survival and neurite regeneration

PMN were prepared from Wistar rat embryos at E14 as described before (Lingor et al., 1999). In detail, donor animals were sacrificed by carbon dioxide intoxication and the embryos extracted from the uterus. The mesencephalic floor was dissected under a stereomicroscope, the meninges were removed and the tissue transferred to ice-cold Hank's balanced salt solution. After trypsinisation (in 750 μl of 0.25% trypsin at 37 °C for 15 min), the tissue was gently triturated using a fire-polished Pasteur pipette for dissociation. After centrifugation at 68 g for 4 min, cells were resuspended in pre-warmed culture medium and