Poly(ADP-ribose) polymerase 1 is a novel target to promote axonal regeneration

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Therapeutic options for the restoration of neurological functions after acute axonal injury are severely limited. In addition to limiting neuronal loss, effective treatments face the challenge of restoring axonal growth within an injury environment where inhibitory molecules from damaged myelin and activated astrocytes act as molecular and physical barriers. Overcoming these barriers to permit axon growth is critical for the development of any repair strategy in the central nervous system. Here, we identify poly(ADP-ribose) polymerase 1 (PARP1) as a previously unidentified and critical mediator of multiple growth-inhibitory signals. We show that exposure of neurons to growth-limiting molecules—such as myelin-derived Nogo and myelin-associated glycoprotein—or reactive astrocyte-produced chondroitin sulfate proteoglycans activates PARP1, resulting in the accumulation of poly(ADP-ribose) in the cell body and axon and limited axonal growth. Accordingly, we find that pharmacological inhibition or genetic loss of PARP1 markedly facilitates axon regeneration over nonpermissive substrates. Together, our findings provide critical insights into the molecular mechanisms of axon growth inhibition and identify PARP1 as an effective target to promote axon regeneration.

poly(ADP-ribose) polymerase | PARP1 | PAR | axon | regeneration

Disability after axonal injury is a poorly treated clinical condition affecting millions worldwide. Neurological impairment has been attributed to a limited capacity of axons to regenerate and restore lost neuronal connectivity and functionality (1). Currently, no therapeutic options exist that promote the regeneration and/or sprouting of axons and enhance recovery of function. Both the intrinsic response of adult neurons to damage and the injury environment contribute to regeneration failure. As CNS neurons mature, their intrinsic capacity for activating growth genes and forming growth cones declines greatly. Studies have shown that reactivating transcriptional programs that are suppressed in mature CNS neurons could promote the growth of adult axons (2). For instance, deletion of PTEN restores mTOR signaling and enhances the regenerative ability of adult corticospinal neurons (3). Similarly, cAMP plays an important role in mediating axon regrowth, via activation of a CREB-directed transcriptional program (4).

In contrast to the intrinsic capacity for axon growth, a number of extrinsic growth-inhibitory proteins and their axonal receptors have been identified within the injury environment. Among them are oligodendrocyte-myelin glycoprotein, myelin-associated glycoprotein (MAG), and Nogo, with their receptors NogoR/p75 (5-7) and paired Ig-like receptor B (8). In addition, astrocyte-produced chondroitin sulfate proteoglycans (CSPGs) are highly up-regulated after CNS injury and contribute to the nonpermissive environment by binding to PTP-σ (9, 10), leukocyte common antigen-related phosphatase (11), and NogoR (12). Finally, ephrins and semaphorins, which are produced by meningeal fibroblasts invading the scar, also restrict the capacity of axons to regenerate (13). Many of these inhibitors signal through RhoA and Rho kinase (ROCK), key molecules in the growth-inhibitory cascades that ultimately converge on the actin and microtubule cytoskeleton, affecting their stability, dynamics, and ability to direct axonal growth (14, 15). Approaches aimed at decreasing the inhibitory extrinsic signals, such as degradation of CSPGs by chondroitinase ABC (16) or genetic deletion of Nogo (17), allow limited axonal growth, but are not sufficient for long-distance axon regeneration, underscoring an unmet need for new targets to promote neural repair.

Poly(ADP-ribose) polymerases (PARPs) are a family of enzymes that consume nicotinamide adenine dinucleotide (NAD^+) to catalyze the addition of ADP ribose polymers (PAR) to their target proteins (PARylation), which modify their functions, interactions, or subcellular localization (18). PARP1, which accounts for 80% of cellular PARP activity, functions as a sensor of DNA strand lesions (19) and in cellular processes such as transcriptional regulation, chromatin dynamics, telomere maintenance, and apoptosis (18). In the context of CNS injury, excessive PARP1 activation contributes to neuronal death through interconnected mechanisms involving NAD^+ depletion, ATP loss, and deregulated PAR synthesis (20). Consistently, the pharmacological inhibition or genetic depletion of PARP1 has been shown to reduce neuronal loss in a variety of experimental models, including ischemic stroke, spinal cord injury, multiple sclerosis, Parkinson's disease, and Alzheimer's disease (21). After injury, the negative impact of PARP1 activation on cellular NAD^+ availability and ATP production—which are critical to axon survival (22) and growth cone motility (23), respectively—may contribute to the lack of axonal regeneration in the CNS. However, although PARP1 is a well-documented mediator of neuronal death and a target for neuroprotection, its role in axonal regeneration after injury is unknown.

Here, we define a previously unidentified role for PARP1 that is independent of genomic DNA damage and neuronal death. We show that, for in vitro models of axonal injury, exposure to

Significance

Damage to axons in the central nervous system typically results in permanent functional deficits; however, the regenerative capacity of injured neurons can be improved by blocking signals from damaged myelin and activated astrocytes, which act as molecular and physical barriers to regeneration. Here, we identify poly(ADP-ribose) polymerase 1 (PARP1) as a previously unidentified and critical mediator of axon growth inhibition. We show that exposure of neurons to diverse growth-inhibiting molecules activates PARP1, resulting in the accumulation of poly(ADP-ribose) in the cell body and axon and limited axonal growth. Accordingly, the pharmacological inhibition or genetic loss of PARP1 markedly facilitates axon regeneration. Together, our findings provide critical insights into mechanisms of axon growth and identify PARP1 as a potent target to reverse neurological disability.

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MAG, Nogo-A, or CSPGs promotes PARP1 activation and the accumulation of PAR in neurons, which leads to axon regrowth failure. Consistently, we demonstrate that pharmacological inhibition or genetic depletion of PARP1 is sufficient to promote regeneration on these distinct nonpermissive substrates. Furthermore, our findings identify PARP1 as a RhoA/ROCK-dependent and -independent component of growth inhibition, which acts locally to regulate axon regrowth through mechanisms that do not require its nuclear activity.

Results

PARP Inhibition Promotes Neurite Outgrowth in Cortical Neurons Exposed to MAG, Nogo-A, or CSPGs. Efforts in the laboratory to identify new targets and strategies to promote neuroregeneration in the CNS led us to investigate whether the inhibition of PARP activity could restore neurite outgrowth in in vitro models of axon growth inhibition. To assess whether PARP inhibitors could overcome myelin-mediated growth inhibition, mouse primary cortical neurons were plated on a monolayer of Chinese hamster ovary (CHO) cells engineered to express the myelin component MAG on their surface (R2M21) or on control CHO cells (R2) (24). Neuronal CHO cocultures were incubated with an effective dose (5 μM; Fig. S1) of one of three structurally distinct PARP inhibitors, PJ34, 4HQ, or 3AB. Criteria to determine the effective dose of PARP inhibitors were minimal toxicity and PAR production, combined with optimal growth on nonpermissive substrates (Fig. S1). After 24 h, cultures were fixed and immunostained for βIII-tubulin, a neuron-specific marker present in axons and dendrites of neurons. Consistent with previous reports (22), neurons grown on MAG-expressing R2M21 cells had significantly shorter neurites than neurons grown on control R2 cells (Fig. 1*A*). By contrast, treatment with PJ34, 4HQ, or 3AB promoted significantly greater neurite outgrowth on MAG-expressing R2M21 cells (Fig. 1*A* and Fig. S2*A*). Neurite lengths were comparable to that of neurons treated with a ROCK inhibitor (Y-27632; 10 μM), a known inducer of neurite outgrowth (22). Importantly, PARP inhibition did not decrease MAG expression in R2M21 cells (Fig. S2*B*).

Another way to model MAG inhibition of neurite growth in vitro is to apply MAG to the bathing medium of neurons that are actively extending neurites (25, 26). In this model, cortical neurons were cultured in poly-D-lysine–coated chamber slides, and soluble MAG (30 μg/mL) was added to the medium, which resulted in significant inhibition of neurite outgrowth within 24 h (Fig. 1*B*). In agreement with our previous observations, treatment of neuronal cultures with PJ34, 4HQ, or 3AB resulted in restoration of neurite growth, despite the presence of MAG. Indeed, when PARP inhibitors were added to the medium of MAG-treated neurons, the average lengths of their neurites were similar to those of neurons treated with a ROCK inhibitor or neurons grown in the absence of MAG (Fig. 1*B* and Fig. S2*C*).

To further address the complexity of the injury environment, cortical cultures were exposed to myelin-derived inhibitor Nogo-A (27). Nogo-A potently inhibited neurite outgrowth, which was significantly rescued by cotreatment with the PARP inhibitors (Fig. 1*C* and Fig. S2*C*).

In addition to myelin debris, CSPGs generated by reactive astrocytes are prominent constituents of the glial scar and contribute to the growth-limiting environment at the site of injury (28). Neurons cultured in chamber slides coated with CSPGs (2 μg/mL) displayed a dramatic inhibition of neurite extension over 24 h of incubation, compared with neurons cultured on poly-D-lysine alone (Fig. 1*D*). However, addition of the PARP inhibitors PJ34, 4HQ, or 3AB restored neurite growth to control levels (Fig. 1*D* and Fig. 2*C*). Treatment with the ROCK inhibitor Y-27632 did not restore neurite growth on CSPGs, confirming the idea that CSPGs activate multiple intracellular signaling pathways (29) (Fig. 1*D*). These findings demonstrate that inhibiting PARP activity allows axon growth in the presence of a variety of inhibitory environmental cues, including MAG, Nogo-A, and CSPGs.

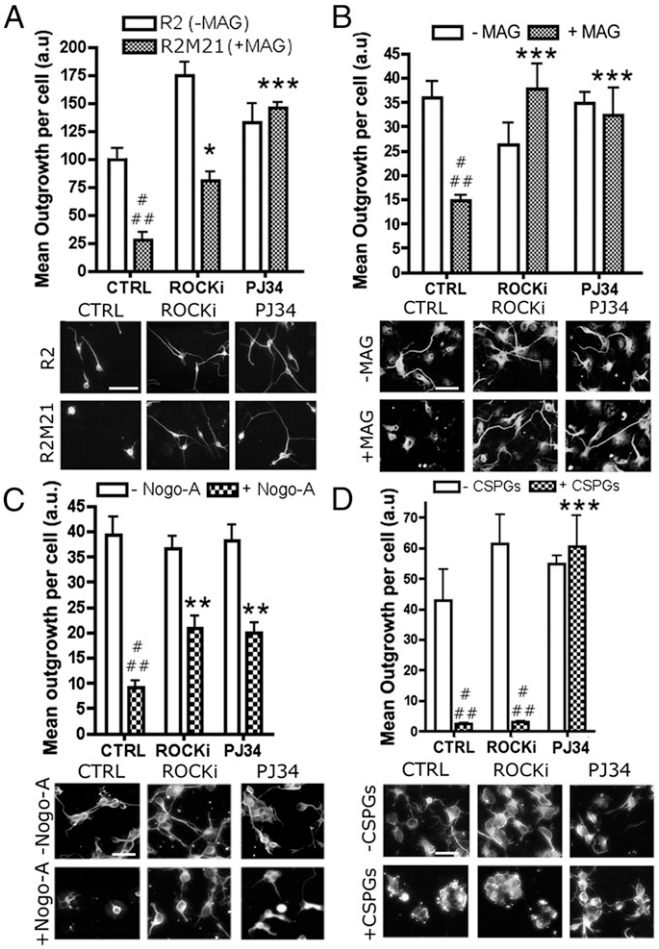

Fig. 1. Pharmacological PARP inhibitors promote neurite outgrowth on MAG, Nogo-A, and CSPGs. Shown is mean outgrowth of primary cortical neurons cocultured for 24 h with control (R2) or MAG-expressing (R2M21) CHO cells (*A*), exposed to MAG (30 μg/mL; *B*), Nogo-A (4 μg/mL; *C*), or CSPGs (2 μg/mL; *D*) in the presence or absence of the RhoA kinase inhibitor (ROCKi; 10 μM) or the PARP inhibitor PJ34 (5 μM). Neurites were identified by neuron-specific TUJ1 immunostaining and measured with Metamorph. $^{\#\#\#}P <$ 0.001 (relative to untreated control); $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (relative to untreated neurons grown in the presence of a growth inhibitor).

PARP Is Activated in Cortical Neurons Exposed to MAG, Nogo-A, or CSPGs. Because PARP inhibition suppresses the axon growth-inhibitory effects of MAG, Nogo-A, and CSPGs, we tested whether exposure to these substrates was sufficient to increase PARP activity in neurons. As such, we examined the formation of PAR, the product of PARP activity, in mouse cortical cultures exposed to MAG, Nogo-A, or CSPGs. After a 24-h exposure, neurons were harvested and lysates immunoblotted for PAR. All three growth-inhibiting molecules induced a robust increase in neuronal PAR levels (Fig. 2*A*), with the potent growth inhibitor Nogo-A being the strongest activator of PAR production (Figs. 1*C* and 2*A*). MAG induction of PAR was consistent with an increased level of enzymatically active PARP1, which is characterized by its auto-PARylation (Fig. 2*B*). Immunofluorescence microscopy confirmed the cell-wide increase in PAR in response to MAG, Nogo-A, and CSPGs. As expected, neuronal accumulation of PAR was significantly decreased by the addition of PJ34, 4HQ, or 3AB (Fig. 2 *C* and *D*), with the more potent

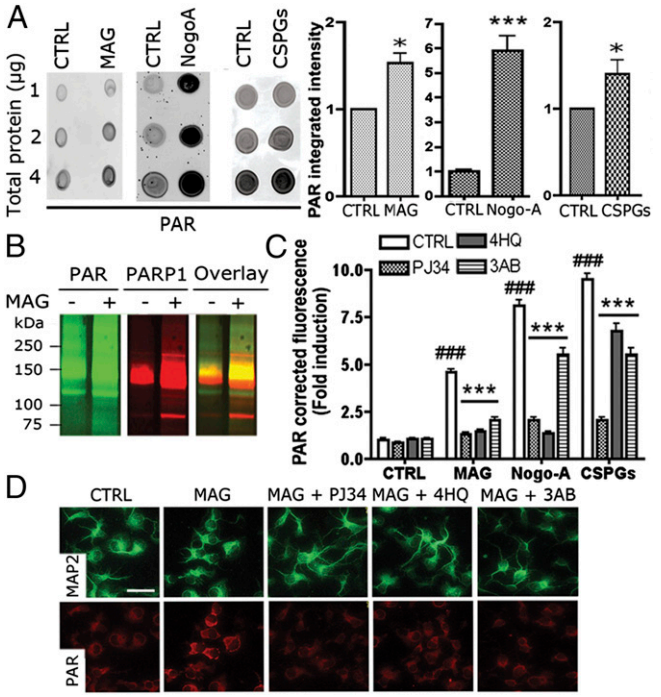

Fig. 2. Neuronal PAR levels are increased after exposure to MAG, Nogo-A, and CSPGs. (*A*) PAR levels in primary cortical neurons exposed to MAG (30 μg/mL), Nogo-A (4 μg/mL), or CSPGs (2 μg/mL) for 24 h. ***$P < 0.001$; *$P < 0.05$ (relative to control). Integrated intensities were measured with Image Studio Lite software. (*B*) PAR (green) and PARP1 (red) expression in response to a 6-h MAG exposure; PARP1 PARylation (yellow) indicates its activation. (*C*) Quantification of PAR immunostaining in response to a 24-h MAG, Nogo-A, or CSPGs exposure, with or without the PARP inhibitors PJ34, 4HQ, or 3AB (5 μM). Corrected total cell fluorescence was measured with ImageJ. $^{###}P < 0.001$ (relative to untreated control); ***$P < 0.001$ (relative to growth inhibitor only). (*D*) Representative images of PAR cellular distribution (red) after exposure to MAG (quantified in *C*). Neurons were identified by MAP2 immunostaining (green).

inhibitor PJ34 displaying the most consistent PARP inhibition across the different conditions. Together, our findings suggest that PARP activation in neurons is part of a signaling cascade that lies downstream of diverse growth-inhibitory cues.

PARP Activation in Response to Growth-Inhibiting Molecules. DNA damage is a well-documented and potent activator of PARP1 (18). To our knowledge, the prospect that exposure of neurons to growth inhibitory molecules—such as MAG, Nogo-A, or CSPGs—induces DNA damage or genomic instability has not been reported. To determine whether DNA damage occurs downstream of growth inhibition signaling, we measured the accumulation of phosphorylated histone H2AX, an early marker of DNA breaks (30). Neurons were exposed to MAG, Nogo-A, CSPGs, or the DNA-damaging agent camptothecin (CPT), which was used as a positive control (30, 31). Unlike CPT, none of the growth-inhibitory molecules induced detectable amounts of H2AX phosphorylation, indicating that PARP1 is activated through a different mechanism (Fig. 3*F*).

Because the intracellular effects of many axon growth-inhibiting molecules are mediated by activation of the small GTP-binding protein RhoA (15, 32), which directly activates ROCK and leads to growth cone collapse, we hypothesized that PARP1 activation could occur through this pathway. To test this hypothesis, we examined the effect of ROCK inhibition on PAR levels in neurons exposed to MAG, Nogo-A, or CSPGs. The analysis of PAR cellular levels revealed that Y-27632 significantly decreased MAG- and Nogo-A-induced PAR (Fig. 3 *A* and *B*). However, treatment with Y-27632 did not decrease CSPGs-induced PAR levels (Fig. 3*C*), which is consistent with our findings that ROCK inhibition does not restore neurite growth on CSPGs (Fig. 1*D*). These results suggest that RhoA/ROCK signaling is necessary for PARP1 activation and neurite growth inhibition downstream of myelin-derived inhibitors, but not CSPGs stimulation. To demonstrate that RhoA/ROCK is sufficient to activate PARP1 and inhibit neurite growth, we electroporated cortical neurons with a plasmidic construct allowing the exogenous expression of high levels of RhoA (Fig. 3*D*). Overexpression of RhoA robustly increased neuronal PAR levels (Fig. 3*E*) and potently inhibited neurite outgrowth within 48 h, which was completely abrogated in the presence of PJ34 (Fig. 3*D*). Together, these results identify PARP1 as a downstream component of RhoA/ROCK-dependent and -independent growth inhibition cascades.

Genetic Deletion of PARP1 Decreases PAR and Promotes Neurite Outgrowth in Cortical Neurons Exposed to MAG, Nogo-A, and CSPGs. The PARP family consists of 17 members, although PARP1 accounts for most cellular PARP activity (18), and is activated in response to MAG (Fig. 2*B*). To confirm that PARP1 is responsible for increased PAR and the target of PARP inhibitors for restoring neurite growth, we compared the effects of MAG, Nogo-A, and CSPGs in cortical

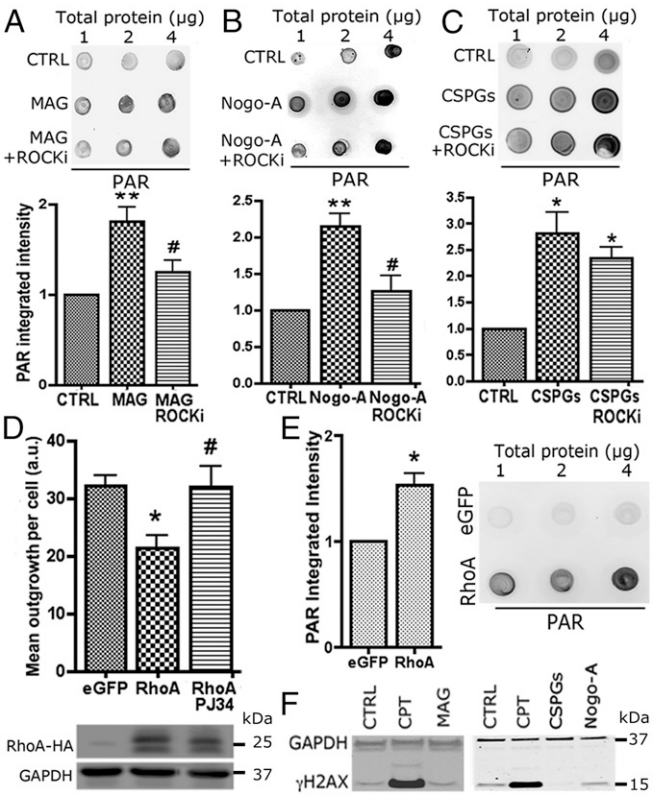

Fig. 3. PARP activation in response to growth-inhibiting molecules. (*A*–*C*) PAR levels in response to a 24-h exposure to MAG (30 μg/mL; *A*), Nogo-A (4 μg/mL; *B*), or CSPGs (2 μg/mL; *C*) in the presence or absence of Rock inhibitor (10 μM; ROCKi). Integrated intensities were measured with Image Studio Lite software. **$P < 0.01$ (relative to CTRL); $^{#}P < 0.05$ (relative to MAG or Nogo-A only). (*D*) Mean outgrowth (*Upper*) of primary cortical neurons overexpressing exogenous RhoA (*Lower*), in the presence or absence of PJ34 (5 μM). RhoA-expressing neurons were identified by HA and TUJ1 double immunostaining, and neurite length was measured with Metamorph. *$P < 0.05$ (relative to eGFP-expressing neurons); $^{#}P < 0.05$ (relative to RhoA only). (*E*) PAR levels in response to exogenous RhoA overexpression. *$P < 0.05$ (relative to eGFP-expressing neurons). (*F*) Histone H2Ax phosphorylation (γH2Ax) in response to a 24-h exposure to MAG, Nogo-A, or CSPGs and a 6-h exposure to the DNA damaging agent CPT (10 μM), which was used as a positive control. GAPDH was used as a loading control.

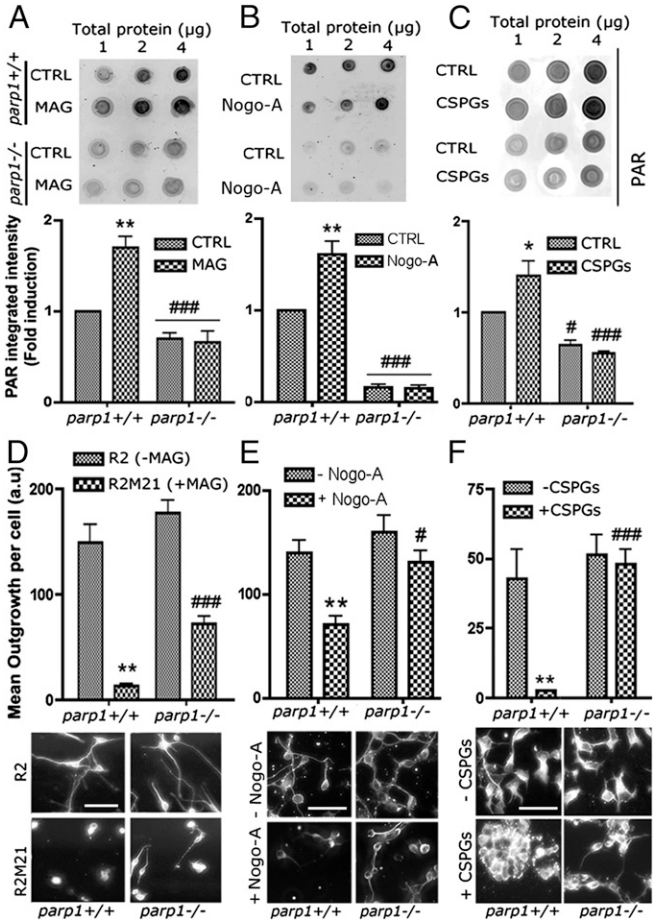

Fig. 4. MAG, Nogo-A, and CSPGs activate PARP1. (*A–C*) PAR levels in *parp1*$^{+/+}$ and *parp1*$^{-/-}$ primary cortical neurons exposed to soluble MAG (30 μg/mL; *A*), Nogo-A (4 μg/mL; *B*), or plated on CSPGs (2 μg/mL; *C*) for 24 h. $^{*}P < 0.05$; $^{**}P < 0.01$ (relative to wild-type CTRL); $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$ (relative to wild-type MAG, Nogo-A, or CSPGs). Integrated intensities were measured with Image Studio Lite software. (*D*) Mean outgrowth of *parp1*$^{+/+}$ and *parp1*$^{-/-}$ primary cortical neurons cocultured for 24 h with control (R2) or MAG-expressing (R2M21) CHO cells. $^{**}P < 0.01$ (relative to wild-type CTRL); $^{\#\#\#}P < 0.001$ (relative to wild-type MAG). (*E* and *F*) Mean outgrowth after a 24-h exposure to Nogo-A (*E*) and CSPGs (*F*). $^{**}P < 0.01$ (relative to wild-type CTRL); $^{\#}P < 0.05$ (relative to wild-type Nogo-A); $^{\#\#\#}P < 0.001$ (relative to wild-type CSPGs).

neurons prepared from wild-type mice (*parp1*$^{+/+}$) and mice lacking PARP1 (*parp1*$^{-/-}$). Consistent with the idea that PARP1 is a major contributor to cellular PARP activity, *parp1*$^{-/-}$ neurons displayed basal PAR levels that were significantly lower than wild type (Fig. 4 *A–C*). Remarkably, exposure of *parp1*$^{-/-}$ neurons to MAG, Nogo-A, or CSPGs failed to increase PAR levels above baseline, suggesting that all three inhibitory molecules predominantly activate PARP1 (Fig. 4 *A–C*). Consistent with the decrease in PAR production, neurons lacking PARP1 extended longer neurites than wild-type when cocultured with MAG-expressing CHO cells (R2M21; Fig. 4*D*) or plated in the presence of Nogo-A (Fig. 4*E*) or CSPGs (Fig. 4*F*).

PARP Inhibition-Induced Neurite Outgrowth Does Not Correlate with Histone PARylation or the Activation of Regeneration-Associated Genes. PARP1 modifies and regulates a variety of nuclear proteins involved in gene regulation, including core histones H2B, H3, and H4, and transcription factors (30, 33). As such, we hypothesized that a potential consequence of PARP1 activation in response to inhibitory cues could be the direct PARylation of histones and the transcriptional repression of genes important for axonal regeneration. To test this hypothesis, PARylation levels of histones H2B, H3, and H4 were determined in neurons after CSPG stimulation, with or without PARP inhibition. Primary cortical neurons were plated on CSPGs in the absence or presence of PJ34 for 24 h, and nuclear extracts were immunoprecipitated by using anti-PAR antibodies. Immunoblot analysis for H2B, H3, and H4 clearly demonstrates that treatment with CSPGs does not increase histone PARylation (Fig. S3 *A* and *B*), despite the increase in neuronal PAR levels (Fig. 2*A*). Additionally, cotreatment with PJ34 did not change baseline levels of histone PARylation (Fig. S3 *A* and *B*).

Because the global PARylation levels of core histones may not be sensitive enough to detect changes that occur at local promoters, we also studied the effect of CSPGs on the expression of known regeneration-associated genes (RAGs). Primary cortical neurons were cultured on CSPGs in the presence of absence of PJ34, and RAG expression was measured at 24 h by quantitative real-time PCR. As can be seen in Fig. S3*C*, none of the RAGs examined (*Atf3*, *Gap43*, *Klf6*, *Klf7*, *Sprr1a*, *Stat3*, and *Tubb3*) were significantly regulated by CSPG stimulation or PJ34, suggesting that PARP inhibition does not alter the intrinsic growth capacity of CNS neurons by activating a regeneration-associated transcriptional program.

PARP Inhibition in the Mature Axon Is Sufficient to Promote Outgrowth in the Presence of CSPGs. Given our findings that PARP inhibition does not alter histone PARylation or the expression of any of the RAGs examined, we questioned whether the reestablishment of axonal growth involves mechanisms restricted to the axon itself. To address this question, we used the leverage of adult dorsal root ganglia (DRG) neuronal cultures grown in compartmentalized microfluidic chambers (Fig. 5*A*), which allow treatments to be applied to the axon alone (24, 34). Neurons were grown in the chambers for 48 h, and the axons growing into the isolated CSPG-containing compartment were treated with either vehicle (PBS) or PJ34. Axonal growth was monitored for 4 h and quantified. In chambers treated with vehicle alone, the presence of CSPGs significantly inhibited DRG axon growth (Fig. 5 *B* and *C*). By contrast, PJ34 added to the fluidically isolated axons significantly increased axonal growth, similar to that of axons growing in the absence of CSPGs (Fig. 5 *B* and *C*). Of note, axonal growth was further increased when PJ34 was applied to both axon and cell body compartments (total, Fig. 5 *B* and *C*). These experiments,

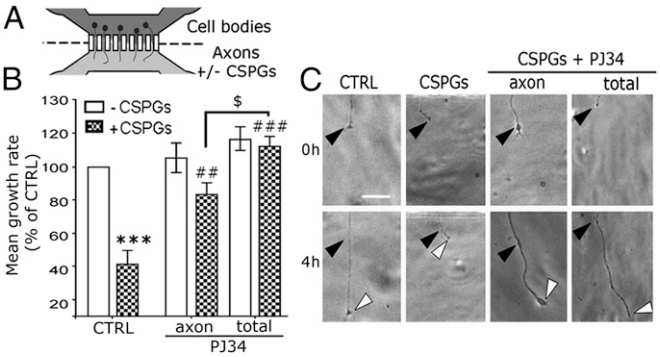

Fig. 5. Pharmacological inhibition of PARP activity promotes DRG axonal outgrowth in the presence of CSPGs. (*A*) Schematic of microfluidic-based culture platform. Volume difference between the somal side and axonal side allows chemical microenvironments to be isolated. (*B*) Growth rate of mouse adult DRG axons exposed to laminin (–CSPGs) or CSPGs in the presence or absence of PJ34 (5 μM) in the axonal compartment only (axon), or in both compartments (total). $^{***}P < 0.001$ (relative to laminin only control); $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ (relative to CSPGs only); $^{\$}P < 0.05$. (*C*) Representative micrographs of axons in the axonal compartment with or without CSPGs, 0 and 4 h after treatment with PJ34. Black arrowheads show axonal growth cones at 0 h. White arrowheads show axonal growth cones at 4 h.

which enable an isolated axonal treatment environment, imply that the effect of PARP inhibition may occur locally in the axons where inhibitory growth signaling is occurring.

PAR Levels Are Increased After Optic Nerve Crush Injury. Given our in vitro findings, we examined PARP activation in the optic nerve (ON) after a crush injury. The ON is widely used to study mechanisms that suppress or promote axon regeneration because it represents a CNS pathway that does not regenerate, and the isolation of ganglion cell (RGC) axons from any surrounding gray matter provides a pure axonal injury when crushing the nerve (35). Immunostaining for PAR, in naïve (n = 3; right ON) and injured (n = 3, left ON) ONs 24h after crush, revealed a significant increase at the site of injury (Fig. S4 *A* and *B*). Interestingly, PAR-positive axons were also clearly visible in the segment proximal to the injury site (Fig. S4*C*, white arrows). By contrast, RGC cell bodies in the retina of injured animals did not show any immunoreactivity for PAR (Fig. S4*D*), suggesting that PAR production is a local response of injured axons in vivo.

Discussion

Strategies aimed at overcoming inhibitory environmental cues, including the neutralization of myelin inhibitors and the degradation of inhibitory components of the glial scar, have been modest at facilitating long-distance regeneration (16, 17). This result may be partly due to the fact that most of these approaches target only one or a few inhibitory molecules at a time, and do not fully address the complexity of the inhibitory environment. Indeed, studies that target multiple components of myelin, CSPGs, and myelin simultaneously, or their receptors have shown a greater enhancement in regeneration than targeting individual components alone (36). Our study identifies exciting and previously unidentified intracellular targets (PARP1 and PAR) that lie downstream of a diverse set of inhibitory signaling molecules. Our results show that PARP1 activity and PAR levels are increased in a dose-dependent fashion in cell bodies and axons of cultured primary neurons exposed to MAG, Nogo-A, or CSPGs (Fig. 2 and Fig. S1). Linking cellular PAR to axon growth, the inhibition of PARP activity, or the genetic loss of PARP1, is sufficient to promote outgrowth in a nonpermissive environment (Figs. 1 and 4). Furthermore, our in vitro studies localize PARP1 activity to the axon, suggesting that PAR production is part of a local signal that determines an axon's growth response to extracellular cues (Fig. 5). These observations were confirmed in an ON crush model, which recapitulates the complexity of CNS axon injury including disruption of axonal tracts and deposition of growth-inhibiting molecules at the site of damage. In agreement with our findings in cultured neurons, we found that PAR accumulated in RGC axons, but not in their cell bodies (Fig. S4). These findings, together with our in vitro mechanistic observations, support the idea that inhibiting PAR production will facilitate axon regeneration in vivo.

In the last few years, a wealth of data linking excessive or dysregulated PARP1 activation to neuronal cell demise and neurodegeneration has emerged. In particular, studies show that downstream of neurological injury, PARP1 can be hyperactivated and promote a type of caspase-independent death termed parthanatos, which is characterized by the mitochondrial release and nuclear translocation of apoptosis-inducing factor AIF (37). Two recent publications describe an additional feature of parthanatos (38, 39), whereby activated PARP1 PARylates and inhibits hexokinase 1 (HK1), an enzyme that reversibly binds to mitochondria through an interaction with the voltage-dependent anion channel and catalyzes the initial step of glycolysis (40). PARylation of HK1 results in a block to glycolysis and a decrease in ATP production that precedes NAD^+ depletion (38, 39). Consistently, pharmacological inhibition or genetic deletion of PARP1 protects against a variety of neurological injuries, including ischemic stroke, spinal cord injury, multiple sclerosis, and Parkinson's and Alzheimer's diseases (41). By contrast, our study describes a model of PARP1 activation that is independent of DNA damage and cytotoxic stress, whereby exposure to growth inhibitory molecules activates PARP1 and promotes PAR accumulation via both RhoA/ROCK-dependent (in response to myelin debris) and RhoA/ROCK-independent (in response to CSPGs) signaling pathways to regulate axonal growth (Fig. 3).

Although this regulated PARP1 activation does not induce cell death, the underlying mechanisms involved in parthanatos, in particular the impact of PARP1 activation on ATP production, may also be common to axon growth inhibition. Motility of growth cones is regulated through the dynamic modulation of tubulin and actin polymerization and uses ∼50% of cellular ATP (42). This high demand in ATP production is ensured by mitochondria and HK1, which are both prominent in the growth cone of adult axons and are essential to support motility (43, 44). Interestingly, blockade of HK1 activity with a synthetic peptide is sufficient to inhibit neurite outgrowth in response to growth factors (45). The inhibitory effect of PARP1 activation on HK1 activity, together with the role of HK1 in axonal outgrowth, is consistent with our observation that growth-inhibitory signals activate PARP1. In agreement with this model, we found that treatment of neurons with pyruvate, which bypasses glycolysis (and HK1), and supports mitochondrial ATP production, allows neurite outgrowth on MAG-expressing CHO cells (Fig. S5*A*), Nogo-A (Fig. S5*B*), and CSPGs (Fig. S5*C*).

In addition to catalyzing the formation of PAR, activated PARP1 consumes intracellular NAD^+ (46). Therefore, it is likely that, in addition to PAR synthesis and HK1 PARylation, lower NAD^+ levels contribute to axonal growth failure and account for the slight disparity between PAR levels and neurite growth that we observe on nonpermissive substrates (Fig. S1). Beyond PARPs, NAD^+ is a substrate for various enzymes whose activity is regulated by its intracellular availability (47, 48). Indeed, it was recently reported that in neurons, PARP1-dependent NAD^+ depletion inhibits the activity of the NAD^+-dependent sirtuin 1 (43), which is critical for axonogenesis and neurite growth (49–52). Likewise, the dynamic modulation of microtubules, essential to growth cone motility highly depends on NAD^+ levels through activation of mitochondrial sirtuin 3 (53). As such, a decline in sirtuin activity due to PARP1-dependent NAD^+ depletion might have a profound impact on neurite growth in an inhibitory environment. Underscoring the importance of axonal NAD^+, many studies have shown that increasing axonal NAD^+ via extra copies of the NAD^+ recycling enzyme NMNAT (22, 54, 55) or providing exogenous NAD^+ or its precursors can markedly delay axon degeneration after transection (22).

Irrespective of the precise mechanisms involved, our findings shed light on a previously unidentified role for PARP1 as a critical mediator of axon growth inhibition downstream of RhoA/ROCK-dependent and -independent signaling cascades (Fig. S6). As such, our work suggests that PARP1 inhibition may be a more robust strategy to treat CNS injury than previously recognized, because it combines neuroprotection with an altered growth state so that neurons are no longer sensitive to multiple growth inhibitory molecules present after injury. Importantly, our observation that PAR rapidly accumulates in axons after ON injury supports the rationale for inhibiting PARP1 to promote regeneration in vivo. From a clinical point of view, PARP1 has garnered significant interest for cancer treatment over the past decade. Several inhibitors are now in phase 1 and 2 clinical trials as chemotherapy sensitizing agents, and the inhibitor olaparib was recently approved by the US food and drug administration and European commission for the treatment of advanced ovarian cancer (56). Along with the established role of PARP1 in neurodegeneration, the findings presented here argue that these selective inhibitors, with their well-defined pharmacodynamics, blood brain barrier permeability, and tolerability, may also be excellent candidate therapeutics for the treatment of neurological injury.

Materials and Methods

All animal surgeries and euthanasia were performed according to Institutional Animal Care and Use Committee guidelines under approved protocols. Male and female animals were used in this study. Embryonic day 15.5-pregnant wild-type CD-1 mice were obtained from Charles River. 129S-Parp1tm1Zqw/J (PARP1-deficient) and 129S1/SvImJ (wild-type) mice were obtained from the Jackson Laboratory and bred in house.

Details of reagents, neuron and cell culture, ON crush, immunoblotting, immunocytochemistry, and real-time quantitative PCR are described in *SI Materials and Methods*. One- or two-way ANOVA followed by the Dunnett's or Bonferroni's post hoc tests were used to measure statistical significance. $P < 0.05$ was considered to be statistically significant. All experiments were performed a minimum of three times.

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