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Effects of Rho Kinase Inhibitors on Grafts of Dopaminergic Cell Precursors in a Rat Model of Parkinson's Disease

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ABSTRACT

In models of Parkinson's disease (PD), Rho kinase (ROCK) inhibitors have antiapoptotic and axon-stabilizing effects on damaged neurons, decrease the neuroinflammatory response, and protect against dopaminergic neuron death and axonal retraction. ROCK inhibitors have also shown protective effects against apoptosis induced by handling and dissociation of several types of stem cells. However, the effect of ROCK inhibitors on dopaminergic cell grafts has not been investigated. In the present study, treatment of dopaminergic cell suspension with ROCK inhibitors yielded significant decreases in the number of surviving dopaminergic neurons, in the density of graft-derived dopaminergic fibers, and in graft vascularization. Dopaminergic neuron death also markedly increased in primary mesencephalic cultures when the cell suspension was treated with ROCK inhibitors before plating, which suggests that decreased angiogenesis is not the only factor leading to cell death in grafts. Interestingly, treatment of the host 6-hydroxydopamine-lesioned rats with ROCK inhibitors induced a slight, nonsignificant increase in the number of surviving neurons, as well as marked increases in the density of graft-derived dopaminergic fibers and the size of the striatal reinnervated area. The study findings discourage treatment of cell suspensions before grafting. However, treatment of the host induces a marked increase in graft-derived striatal reinnervation. Because ROCK inhibitors have also exerted neuroprotective effects in several models of PD, treatment of the host with ROCK inhibitors, currently used against vascular diseases in clinical practice, before and after grafting may be a useful adjuvant to cell therapy in PD. STEM CELLS TRANSLATIONAL MEDICINE *2016;5:804–815*

SIGNIFICANCE

Cell-replacement therapy is one promising therapy for Parkinson's disease (PD). However, many questions must be addressed before widespread application. Rho kinase (ROCK) inhibitors have been used in a variety of applications associated with stem cell research and may be an excellent strategy for improving survival of grafted neurons and graft-derived dopaminergic innervation. The present results discourage the treatment of suspensions of dopaminergic precursors with ROCK inhibitors in the pregrafting period. However, treatment of the host (patients with PD) with ROCK inhibitors, currently used against vascular diseases, may be a useful adjuvant to cell therapy in PD.

INTRODUCTION

Consistent with the antiapoptotic properties of Rho kinase (ROCK) inhibition [1], ROCK inhibitors have been used in a variety of applications associated with cell dissociation in the process of stem cell research, such as passaging, expansion, cryopreservation, gene transfer, induction of differentiation, and cell sorting. It has been suggested that ROCK inhibitors may constitute an important tool for improving the handling of human induced pluripotent stem (iPS) cells and embryonic stem (ES) cells [2, 3], mouse ES cell-derived neural precursors [4], mouse intestinal stem cells [5], human keratinocytes [6], and bone marrow mesenchymal cells [7]. In contrast, ROCK inhibitors have detrimental effects on the expansion and survival of both fresh and cryopreserved cells [8–11].

One promising therapeutic approach for Parkinson's disease (PD) is cell-replacement therapy, in which dopaminergic precursors are grafted into the striatum to restore the lost dopaminergic neurotransmission. However, many questions, including the use of sources of cells other than primary tissue (i.e., ES cells or iPS cells), must be addressed before widespread application of the treatment is possible [12–16]. A major limiting factor is the poor survival of grafted dopaminergic

neurons. Furthermore, axonal outgrowth from the grafted neurons, which is important for clinical efficacy, is often poor, particularly outgrowth from neurons obtained from sources other than primary tissue. Independently of the cell source, strategies for improving survival of grafted cells and graft-derived dopaminergic innervation are therefore required [17, 18].

ROCK inhibition has antiapoptotic and axon-stabilizing effects in damaged neurons [19]. ROCK inhibitors also show anti-inflammatory properties via their effects on migration, phagocytosis, and several other mechanisms involved in the inflammatory response in the central nervous system (CNS) and other tissues [20, 21]. ROCK signaling is also an important mediator in many angiogenic processes, including migration, survival, and permeability of endothelial cells [22]. All of these mechanisms (reviewed by Labandeira-Garcia et al. [23]) play critical roles in the growth and functional effects of dopaminergic grafts. Furthermore, recent studies in PD models have shown that ROCK inhibitors protect against dopaminergic neuron death and axonal retraction induced by dopaminergic neurotoxins [23]. However, no studies have yet considered the effects of ROCK inhibitors on dopaminergic cell grafts. The current study investigated this aspect by treating suspensions of dopaminergic precursors destined for grafting and host animals (rats lesioned with the dopaminergic neurotoxin 6-hydroxydopamine [6-OHDA]) with ROCK inhibitors. We also used primary cultures of dopaminergic neurons to clarify the mechanisms involved in the observed effects.

Materials and Methods

In Vitro and In Vivo Treatment With ROCK Inhibitors

In vitro, we tested the effects of Y-27632, which is the ROCK inhibitor commonly used for improving handling of different types of cells. In rats, we tested the effects of fasudil, which is the ROCK inhibitor commonly used for in vivo studies. Fasudil is the only ROCK inhibitor approved for clinical use (approved in Japan since 1995). Y-27632 is a potent ROCK inhibitor, which is also active after oral administration, but it is metabolized rapidly and its brain penetration might be too low to achieve therapeutical levels for CNS diseases. However, Y-27632 is a more potent inhibitor and exhibits more favorable selectivity profile than fasudil for in vitro studies [24]. We performed additional in vitro experiments with fasudil to confirm major results obtained with Y-27632 and to exclude any possible difference between the effects of both inhibitors.

In Vivo Experiments: Experimental Design

Eight-week-old female Sprague-Dawley rats were used. All experiments were carried out in accordance with the European Communities Council Directive 2010/63/EU and Directive 86/609/EEC and were approved by the corresponding committee at the University of Santiago de Compostela. Rats were subjected to a prelesion cylinder test (discussed later in this paragraph). Rats were then subjected to maximal unilateral dopaminergic denervation with 6-OHDA (discussed later in this paragraph). One month after injection with 6-OHDA, rats with maximal lesions (total or almost total dopaminergic depletion; $>$90%) were selected in the rotometer test after administration of apomorphine (0.05 mg/kg subcutaneously; Sigma-Aldrich, St. Louis, MO, https://www.sigmaaldrich.com/) [25]. One week after the rotometer test, the rats with maximal lesions (n = 34) were subjected to a postlesion cylinder test and were again tested in the rotometer after administration of D-amphetamine (5 mg/kg in saline intraperitoneally; Sigma-Aldrich). One week after the last postlesion behavioral test, the rats were randomly divided into 2 experimental groups to investigate the effect of the ROCK inhibitors. Rats in group A (n = 17; 3 subgroups) were used to study the effect of ROCK inhibitor Y-27632 on survival of grafted fetal ventral mesencephalon (VM) cell suspensions. One subgroup of rats (A1, Y-27632 group; n = 6) received a VM cell suspension containing 30 μM Y-27632 (Sigma-Aldrich). The dose of Y-27632 was selected in accordance with the results of previous studies [26–32]. Another subgroup of rats (A2, control group; n = 6) was grafted with the same VM cell suspension containing vehicle. The third subgroup (A3, sham group; n = 5) was sham grafted with vehicle. Rats in group B (n = 17; 3 subgroups) were used to test the effect of the ROCK inhibition in the host rats on survival of grafted VM precursors. Rats in subgroup B1 (fasudil group; n = 6) were grafted with a cell suspension from the VM and were also administered the ROCK inhibitor fasudil (30 mg/kg per day; LC Laboratories, Woburn, MA, http://www.lclabs.com/) in their drinking water from 15 days before receiving the cell implants until they were killed. The dose of fasudil was selected in accordance with the results of previous studies [27, 33–35]. Rats in subgroup B2 (control; n = 6) were grafted with the same VM cell suspension and administered vehicle in their drinking water during the same period as subgroup B1. Rats in subgroup B3 (sham group; n = 5) were sham grafted with vehicle and treated orally with vehicle. Six weeks after grafting, the rats were again tested in the cylinder and the rotometer and were then killed for histological analysis.

6-OHDA Lesion and Transplantation Surgery: Behavioral Testing of Lesion Efficacy and Graft Survival

Unilateral lesions of the dopaminergic system were performed by injection into the right medial forebrain bundle of 12 μg of 6-OHDA hydrobromide (Sigma-Aldrich). The stereotaxic coordinates were 3.7 mm posterior to the bregma, $-$1.6 mm lateral to midline, and 8.8 mm ventral to the skull [36]. Rats in subgroups A1, A2, B1, and B2 (i.e., rats transplanted with primary fetal tissue) received intrastriatal injections of cell suspensions prepared from fetal (13 days of gestation, E13) VM. The pieces of VM were dissected out and incubated in 0.1% trypsin (Sigma-Aldrich), 0.05% DNase (Sigma-Aldrich), and Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific Life Sciences, Waltham, MA, http://www.thermofisher.com) for 20 minutes at 37°C. The tissue was then rinsed in DNase/DMEM and mechanically dissociated to obtain a cell suspension with a final concentration of 150,000 cells/μl. Rats in subgroup A1 were grafted with a VM cell suspension containing 30 μM Y-27632, which was added after cell dissociation. A total of 3 μl of cell suspension from primary fetal tissue was administered to each rat at a single injection site (anterior to bregma, 0.6 mm; lateral to midline, $-$2.7 mm; ventral to the skull, 4.5 mm).

The efficacy of the lesion and graft survival was evaluated by the rotometer test (i.e., maximally lesioned rats) [25] and the cylinder test [37], and they were confirmed by subsequent immunohistochemistry (see discussion later in text). Turning behavior was monitored for 90 minutes after injection of D-amphetamine or for 60 minutes after injection of apomorphine. Spontaneous forelimb use was assessed by an observer blinded to the identity of the animals, who scored the number of forelimb contacts with

the cylinder wall, recording a total number of 20 touches for each animal. Forelimb asymmetry, assessed as use of the left impaired paw, was expressed as percentage of the total number of touches.

Immunolabeling and Quantitative Histological Analysis

At 6 weeks after grafting, the animals were perfused with 4% paraformaldehyde and the brains were cut into 40-μm sections. Series of free-floating sections were processed for a mouse monoclonal antibody to tyrosine hydroxylase (TH; dopaminergic marker; 1:10,000; Sigma-Aldrich) or a mouse monoclonal antiserum to rat endothelial cell antigen-1 (RECA-1; marker of rat endothelial cells; 1:2,000; AbD Serotec, Oxford, U.K., https://www.abdserotec.com). The labeling was visualized by using 3,3′diaminobenzidine (DAB; Sigma-Aldrich). Additional series of sections containing the transplants were processed for double immunolabeling against TH and RECA-1 using the immunoperoxidase technique.

Quantification of TH-immunoreactive (-ir) neurons or RECA-1-positive microvessels in grafts was carried out in every fourth section to cover the entire graft from the rostral tip to the caudal end. Sampling was carried out with the Computer Assisted Stereological Toolbox (CASTGrid system; Olympus, Ballerup, Denmark, http://www.olympus.dk/). The total numbers of TH-positive neurons and RECA-1-positive microvessels in the grafts were calculated according to the optical fractionator equation [38]. The graft volume was estimated according to Cavalieri's method [39]. The striatal graft-derived reinnervation area (TH-ir area surrounding the graft) was measured by using the CAST-Grid system. At least four sections through the grafted striatum of each rat were measured and expressed in mm^2. The density of the TH-ir fibers in the graft-derived reinnervation area was estimated as the optical density of striatal TH-ir with the aid of Image 1.55 image analysis software (National Institutes of Health, Bethesda, MD, http://imagej.nih.gov). At least four sections through the graft of each animal were measured, and for each section optical density was corrected by subtraction of background as observed in the corpus callosum.

In Vitro Experiments: Primary Mesencephalic Cultures and Cell Suspensions

VM tissue was dissected from rat embryos (E13) and mechanically dissociated, and the resulting cell suspension was treated as described previously [40]. The primary mesencephalic cell cultures were maintained in a humidified CO_2 incubator (5% CO_2, 37°C) for 7 days in vitro (DIV). To study the possible involvement of ROCK in dopaminergic survival, primary cultures were treated with the ROCK inhibitors Y-27632 or fasudil under different experimental conditions. In a first group of cultures (group 1), Y-27632 or fasudil (10 or 30 μM) was added 1 hour before cell dissociation (i.e., previously to obtain a single cell suspension from VM pieces). Some cultures (group 2) were treated with Y-27632 or fasudil 1 hour before dissociation and after dissociation (i.e., previously to obtain a single cell suspension and added into the culture medium at plating). Cultures of group 3 were treated with Y-27632 or fasudil only after dissociation (i.e., at plating). Group 2 and 3 cell cultures were maintained for 2 DIV in the presence of Y-27632 or fasudil (i.e., until the culture medium was replaced with fresh medium). Some primary cultures (group 4) were treated with Y-27632 or fasudil (30 μM) on 7 DIV for 24 hours. Finally, a group of cell suspensions were treated with Y-27632 (30 μM) or fasudil (30 μM) for up to 24 hours, and cell survival was estimated before plating by methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay as follows. After treatment with ROCK inhibitors Y-27632 (30 μM) or fasudil (30 μM) for 30 minutes or 2, 4, or 24 hours, cells were incubated with MTT (1 mg/ml) for 4 hours at 37°C. After removing the MTT in the supernatant, the formazan crystals were dissolved in acidic isopropanol and quantified using a plate reader at 570 nm (Tecan Infinite M200, Grödig, Austria, http://lifesciences.tecan.com).

Immunocytochemistry and Cell Counts

Cultures were fixed with 4% paraformaldehyde and incubated with a mouse monoclonal anti-TH (1:30,000). The labeling was revealed with DAB. TH-ir cells, observed by phase-contrast microscopy (magnification, $\times$100; Eclipse, Nikon, Tokyo, Japan, https://www.nikoninstruments.com), were counted in 5 randomly chosen longitudinal and transverse microscopic fields along the diameter of the culture dish. The microscopic field was defined by a 0.5-cm $\times$ 0.5-cm reticule (i.e., a total of 1.25 cm^2). The results from at least 3 different experiments were recorded. The results were normalized to the counts of the control group in the same experiment (i.e., expressed as a percentage of the control group counts). In addition, neurons from control cultures and mature cultures treated with fasudil or Y-27632 (30 μM, on 7 DIV for 24 hours) were randomly selected, and the lengths of primary processes of every neuron examined were measured with the aid of NIH-Image analysis software. A minimum of 75 processes from 3 independent experiments was measured per treatment group. Clusters of cells in which primary processes were not clearly distinguished were excluded, and the length of a primary process was defined as the distance from the soma to the tip of the longest branch [26].

Statistical Analysis

Two-group comparisons were carried out by using the Student's *t* test. Multiple comparisons were analyzed by one-way analysis of variance followed by Bonferroni post hoc test. The normality of populations and homogeneity of variances were tested before each analysis of variance. The results are presented as means $\pm$ SEM. Differences were considered statistically significant at $p < .05$. All statistical analyses were performed with SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, https://systatsoftware.com/).

RESULTS

ROCK Inhibition of Cell Suspension Destined for Grafting: Effects on Dopaminergic Neuron Survival and Striatal Reinnervation

In vivo analysis of amphetamine-induced rotational response 6 weeks after grafting showed no significant differences in post-grafting rotations of sham-operated animals and of rats after 6-OHDA lesion (1,257 $\pm$ 222.8 turns and 1,290.8 $\pm$ 161.8 turns, respectively). However, net rotations decreased markedly in rats that received control cell suspensions and rats that received cell suspensions treated with the ROCK inhibitor Y-27632 (343.2 $\pm$ 68.9 turns and 423.2 $\pm$ 45.1 turns, respectively) relative to the values recorded for these groups after lesion (1,479.9 $\pm$ 234.8 turns and 1,309 $\pm$ 112.1 turns, respectively). This indicated that a significant number of grafted dopaminergic neurons had

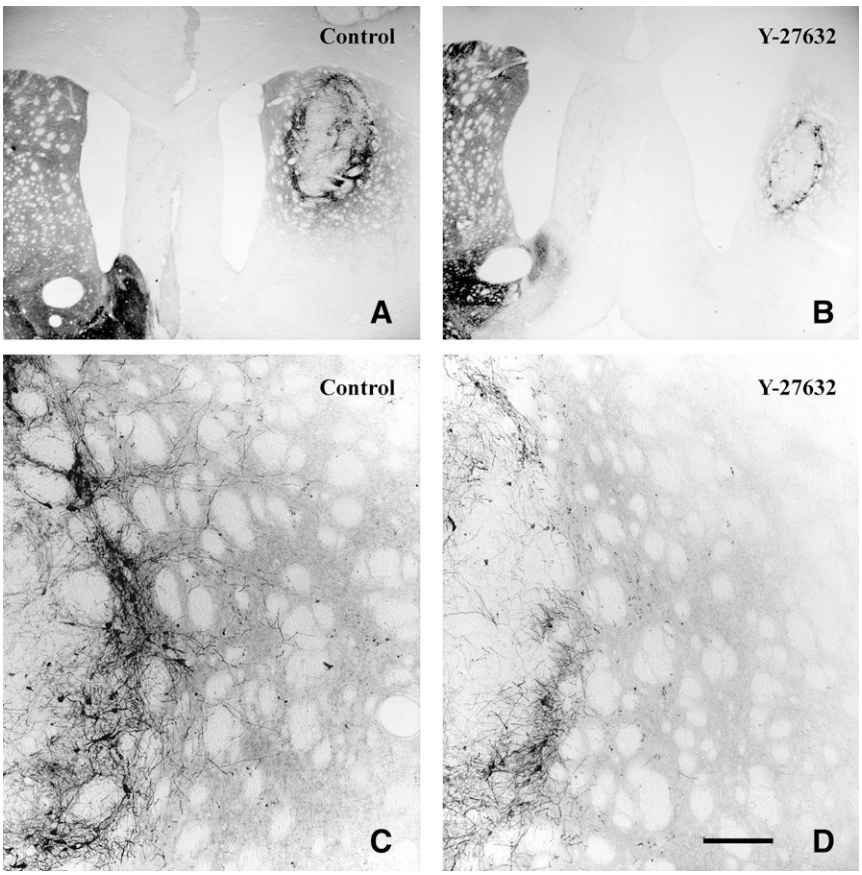

Figure 1. Photomicrographs of intrastriatal ventral mesencephalic (VM) grafts. Immunoreactivity (-ir) against tyrosine hydroxylase (TH) in rats subjected to unilateral dopaminergic denervation and grafting with VM cell suspensions (i.e., control; **A, C**), or with VM cell suspensions treated with the Rho kinase inhibitor Y-27632 (30 μM; **B, D**). In control grafts, numerous TH-ir neurons are located in the periphery of the grafts, and TH-ir fibers innervate the striatal area surrounding the grafts (i.e., reinnervation area). However, the number of TH-ir cells and the density of TH-ir fibers in the reinnervated area were significantly lower in the Y-27632-treated grafts. Scale bar = 1.5 mm **(A, B)** and 200 μm **(C, D)**.

survived. However, it is known that even a relatively low number of surviving dopaminergic neurons can totally reverse the rotational asymmetry, and a postmortem morphological analysis was necessary to evaluate possible differences in graft development. In the cylinder test, no significant recovery was observed 6 weeks after lesion in sham-operated animals related to previous values (i.e., 4% $\pm$ 1.9% and 5% $\pm$ 3.2% of left paw use, respectively; subgroup A3). However, use of the impaired paw improved significantly in rats that received the untreated VM cell suspension (i.e., 11% $\pm$ 5.1% and 39% $\pm$ 8.4% of left paw use before and after grafting, respectively; subgroup A2). Animals that received the cell suspension treated with Y-27632 did not show a significant improvement in values of impaired paw use compared with the pregrafting values (17% $\pm$ 5.2% and 31% $\pm$ 10.1% of left paw use; subgroup A1).

As observed in previous immunohistochemical studies, TH-ir neurons were not evenly distributed within the graft but were usually grouped in patches located at the periphery of the graft, whereas the central area of the grafts was usually TH-negative (Fig. 1A). Although the distribution of TH-ir cells in graft of rats that received cells suspensions treated with Y-27632 was similar to that in control grafts, the number of dopaminergic cells was significantly lower than the standard reference (i.e., control group; 5,405.3 $\pm$ 880.8 TH-positive cells in control group and 2,117.5 $\pm$ 388.9 TH-positive cells in Y-27632-treated group) (Figs. 1, 2A). The average graft volume in the Y-27632-treated group was lower than that observed in animals grafted with non-treated cell suspensions (i.e., control group), although the difference was not statistical significant (0.306 $\pm$ 0.089 mm^3 in Y-27632-treated group and 0.448 $\pm$ 0.097 mm^3 in control group). Grafts in rats that received transplants of control VM precursors extended numerous processes into the host striatum, and the graft was surrounded by a TH-ir area (i.e., reinnervation area) (Fig. 1A, 1C). However, in the Y-27632-treated group (Fig. 1B, 1D), both the reinnervated area and the striatal TH-ir fiber density were significantly lower than in control grafted animals (a decrease of about 50% in the reinnervation area and about 60% in the striatal TH-ir density) (Figs. 1, 2B, 2C).

ROCK Inhibition in the Host Rats: Effects on Dopaminergic Neuron Survival and Striatal Reinnervation

In the rats subjected to maximal dopaminergic denervation with 6-OHDA, evaluation of amphetamine-induced rotational behavior displayed intense rotation toward the denervated side (1,065.8 $\pm$ 111.8 turns for control-grafted group, 1,312 $\pm$ 185.9 turns for the group that then was treated with fasudil, and 1,290.4 $\pm$ 161.8 turns for sham group). Six weeks after grafting, evaluation of the amphetamine-induced rotational behavior

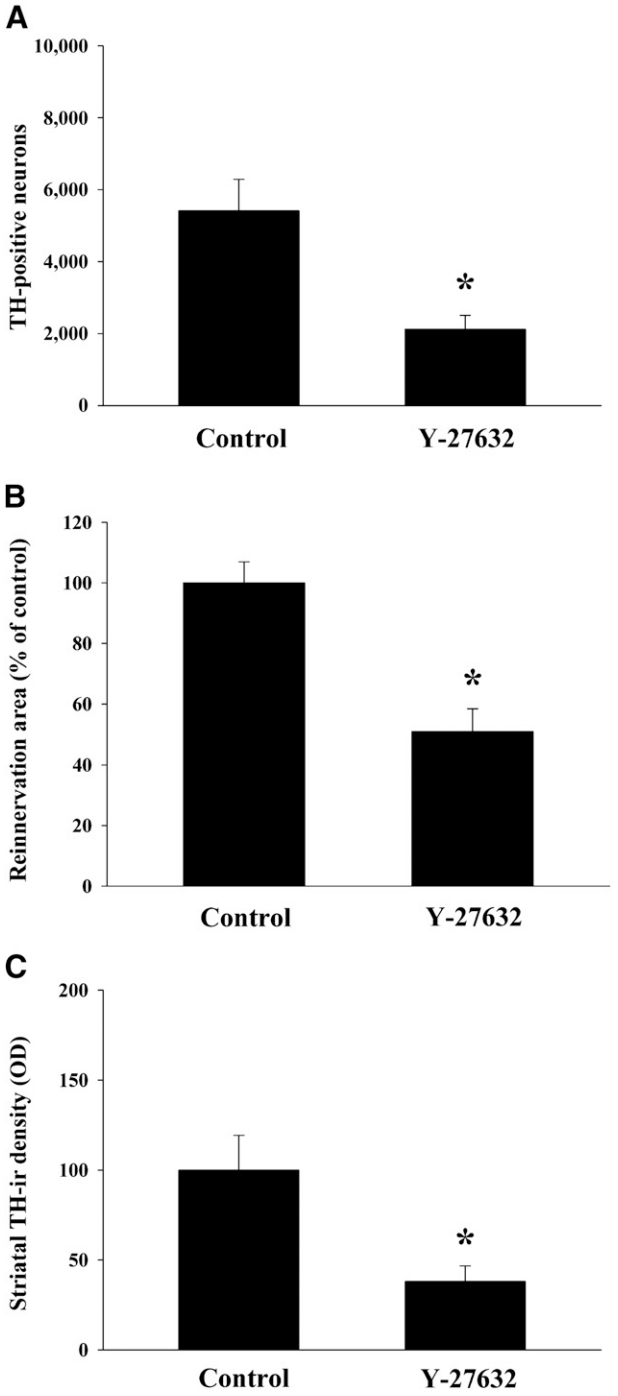

Figure 2. Quantitative histological analysis of intrastriatal grafts of ventral mesencephalic (VM) cell suspensions treated and not treated with the Rho kinase inhibitor Y-27632 (30 μM). **(A):** Number of dopaminergic neurons (i.e., TH-positive neurons). **(B):** Reinnervation area (i.e., striatal host area innervated by graft-derived TH-ir fibers). **(C)**: Density of TH-ir fibers in reinnervated area estimated as a optical density and expressed as a percentage of the value obtained in the control group. Data represent mean $\pm$ SEM. *, $p < .05$ relative to control-grafted rats (Student's *t* test). Abbreviations: OD, optical density; TH, tyrosine hydroxylase; TH-ir, immunoreactive tyrosine hydroxylase.

revealed that the intense ipsiversive rotation observed after the 6-OHDA lesion was significantly reduced in animals that received a suspension of VM precursors (655 $\pm$ 161.8 turns for the control/untreated grafted group and 587.5 $\pm$ 167.9 turns for the fasudil-treated group), whereas no significant decrease in rotational behavior was observed in sham-grafted rats (1,330.4 $\pm$ 136.2 turns). Similarly, in the cylinder test both transplanted groups showed significant recovery of left paw use relative to the pregrafting (i.e., postlesion) values (10% $\pm$ 4.1% and 43.8% $\pm$ 7.7% in the control grafted group; 15% $\pm$ 6.1% and 56.3% $\pm$ 15.6% in the fasudil-treated group). No significant improvement was observed in sham-operated animals relative to the postlesion values (9% $\pm$ 4% and 5% $\pm$ 1.6% of left paw use).

Rats with control VM implants showed numerous TH-ir cells concentrated in the periphery of the graft. The distribution of TH-ir cells in grafts from animals treated with fasudil was similar to that observed in the control group. The total number of TH-ir neurons was apparently higher in fasudil-treated rats, although the increase was not statistically significant (6,205.8 $\pm$ 947.1 TH-positive cells in the control group and 7,156.7 $\pm$ 806.5 TH-positive cells in the fasudil-treated group) (Figs. 3, 4A). The average graft volume in the fasudil-treated group was not significantly different from that observed in untreated rats (i.e., control group; 0.441 $\pm$ 0.065 mm^3 in the fasudil-treated group and 0.486 $\pm$ 0.038 mm^3 in the control group). In both control and fasudil-treated groups, numerous processes grew from the grafts into the host striatum, producing a TH-ir area (i.e., reinnervation area) surrounding the graft. Interestingly, in the fasudil-treated group, the reinnervated area was significantly larger than that in control grafted rats (around 80% increase) (Figs. 3C, 3D, 4B). There was also a significant increase in the density of striatal TH-ir fibers in the reinnervated area in the fasudil-treated group relative to the control group (an approximately 70% increase) (Fig. 4C).

Effect of ROCK Inhibition on Graft Microvessels

Control grafts showed tubular structures positive for RECA-1, forming widely ramified networks that covered the graft (Fig. 5A, 5B). When VM cell suspensions were treated with the ROCK inhibitor Y-27632 before grafting, a significant decrease in the number of blood microvessels was observed relative to the control group (259.7 $\pm$ 7.6 vessels/mm^2 in control group and 203.9 $\pm$ 19.1 vessels/mm^2 in Y-27632-treated group) (Fig. 5C–5E). However, treatment of the host with the ROCK inhibitor fasudil had no significant effect on the number of RECA-1-positive vessels in the graft (250.2 $\pm$ 28.4 vessels/mm^2 in the control group and 242.1 $\pm$ 15.5 vessels/mm^2 in the fasudil-treated group) (Fig. 5C, 5F, 5G).

Effect of ROCK Inhibition on Dopaminergic Neuron Survival in Vitro

Control cultures showed numerous TH-ir neurons and the average number of TH-positive cells in a control culture dish was 5,059 $\pm$ 226. However, the number of TH-ir cells decreased significantly (a decrease of approximately 60%) when the mesencephalic cell suspension was treated with 30 μM Y-27632 after dissociation (i.e., at plating) (Figs. 6A, 7A, 7B). Cultures treated with lower dose of Y-27632 (10 μM) showed a low, nonsignificant decrease in the number of TH-ir cells as control cultures. To confirm that this effect was related to the timing of treatment, primary cultures were treated with the ROCK inhibitor Y-27632 (30 μM) for 24 hours at 7 DIV. In this case, no significant differences in the number of dopaminergic neurons were observed in cultures treated with Y-27632 relative to the control group (Figs. 6A, 7D). To study whether

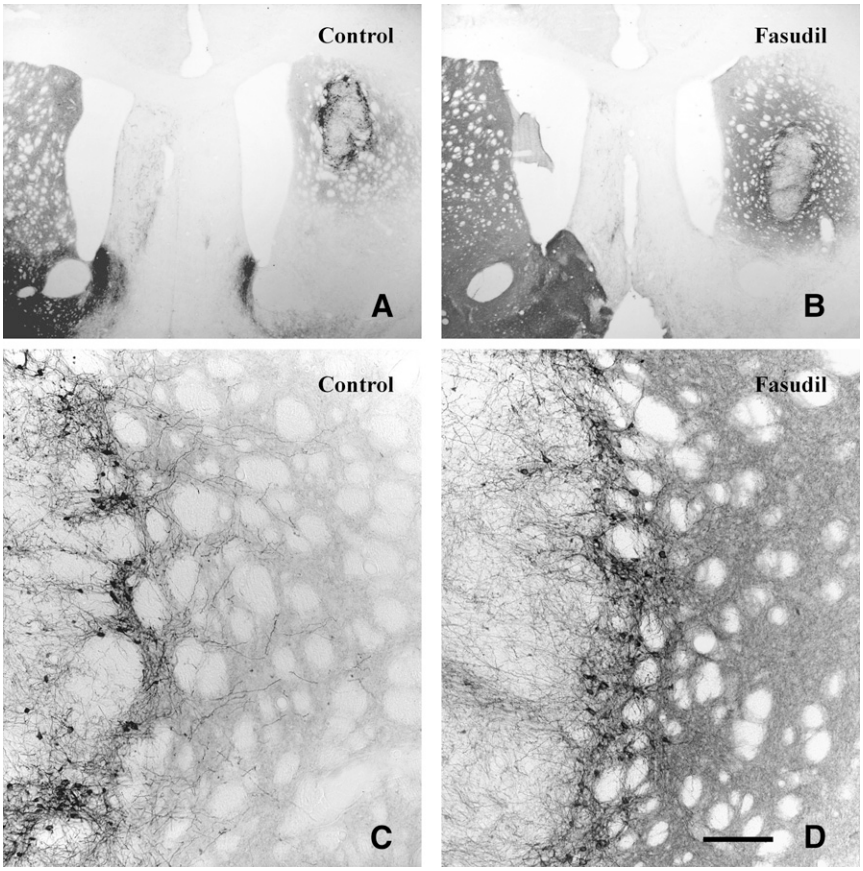

Figure 3. Photomicrographs of intrastriatal ventral mesencephalic (VM) grafts. Immunoreactivity (-ir) against tyrosine hydroxylase (TH) in rats subjected to unilateral dopaminergic denervation and grafting with VM cell suspensions (i.e., control; **A, C**), or with VM cell suspensions transplanted in rats treated with the Rho kinase inhibitor fasudil **(B, D)**. Numerous TH-ir neurons are located in the periphery of the grafts, and TH-ir fibers innervate the striatal area surrounding the grafts (i.e., reinnervation area) both in control and fasudil-treated groups. However, in the fasudil-treated group the reinnervated area and the density of striatal TH-ir fibers were significantly higher than in the control group. Scale bar = 1.5 mm **(A, B)** and 200 μm **(C, D)**.

treatment with ROCK inhibitors affects dissociation-induced cell death, cells were treated with Y-27632 either before (only) or before and after dissociation. Treatment with 30 μM Y-27632 before cell dissociation or before and after dissociation significantly reduced the number of TH-ir cells in culture. However, the number of TH-ir cells was similar to the control group in cultures treated with 10 μM Y-27632 added before (only) or before and after dissociation (Figs. 6B, 7C). Furthermore, some cultures were treated with fasudil to confirm the effects of Y-27632 and to exclude any possible particular effect of Y-27632 on dopaminergic neuron viability (Fig. 6C, 6D). Similar to observations after treatment with Y-27632, the number of TH-ir cells decreased significantly when the mesencephalic cell suspension was treated with 30 μM fasudil before and/or after (i.e., at plating) cell dissociation, whereas a lower dose of fasudil (10 μM) induced a low, nonsignificant decrease in the number of TH-ir cells. However, cultures treated with fasudil (30 μM) for 24 hours at 7 DIV did not show significant differences in the number of dopaminergic neurons relative to the control group. Consistent with previous studies [26], no significant change in the length of dopaminergic neurites was observed in mature cultures (i.e., on 7 DIV) treated with the ROCK inhibitor Y-27632 (control: 100% $\pm$ 5.75%; Y-27632: 97.87% $\pm$ 7.06%) or fasudil (control: 100% $\pm$ 4.51%; fasudil: 109.77% $\pm$ 4.44%) relative to untreated cultures (i.e., controls).

Cell suspensions treated (30 minutes–24 hours) with Y-27632 (30 μM) or fasudil (30 μM) and analyzed for cell viability did not show any significant difference in cell survival (as measured by the MTT assay) relative to untreated controls (Fig. 6E, 6F).

Discussion

Cell Therapy in Parkinson's Disease: Effects of ROCK Inhibition

The results of open-label clinical trials [41, 42] and double-blind, placebo-controlled trials [43, 44] indicate that replacement of dopamine-producing neurons may be useful, but also that many questions must be addressed before widespread application of the treatment is possible [45–47]. Independently of the cell source, strategies to improve axonal growth and survival of grafted cells are required. Data from previous studies suggest that ROCK inhibitors may improve the survival of grafted neurons and axonal outgrowth. However, we observed different effects after treatment of cell suspensions of dopaminergic precursors or host animals with ROCK inhibitors. In the present study, all grafted rats showed significant graft-induced recovery in rotational behavior and the cylinder test. However, it is known that a small number

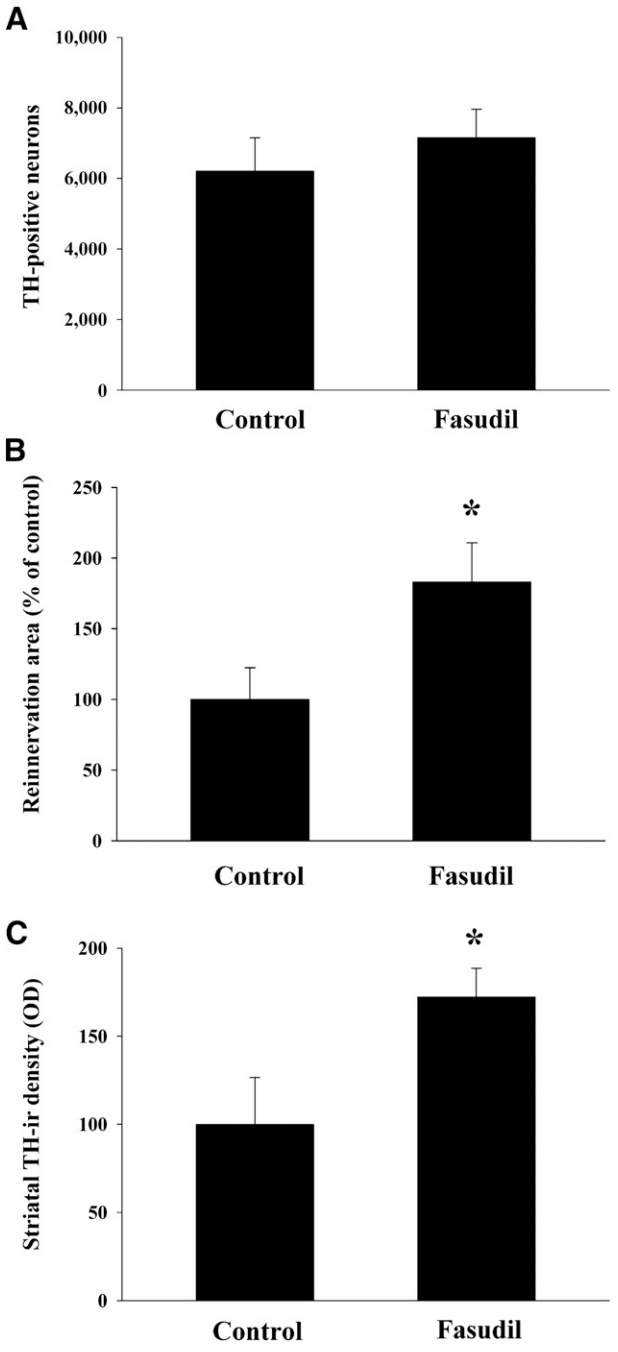

Figure 4. Quantitative histological analysis of intrastriatal grafts of ventral mesencephalic cell suspensions (i.e., control) in rats treated and not treated with the Rho kinase inhibitor fasudil. **(A):** Number of dopaminergic neurons (i.e., TH-positive neurons). **(B):** Reinnervation area (i.e., striatal host area innervated by graft derived TH-ir fibers). **(C):** Density of TH-ir fibers in reinnervation area estimated by optical density, expressed as a percentage of the value obtained in the control group. Data represent mean ± SEM. *, $p < .05$ relative to control-grafted rats (Student's *t* test). Abbreviations: OD, optical density; TH, tyrosine hydroxylase; TH-ir, immunoreactive tyrosine hydroxylase.

of surviving dopaminergic neurons can reverse the rotational behavior [48, 49]. More sophisticated behavioral methods must be found in the future to demonstrate functional differences between grafts in rats [50].

ROCK Inhibition and Dopaminergic Cell Death

It has been suggested that dissociation of neural precursor cells elicits an intrinsic pathway of cell death that is at least partially mediated through the ρ/ROCK pathway, and ROCK inhibitors rescue these cells, as well as iPS and ES cells, from dissociation-induced apoptosis and from passing and cryoinjury [2, 4, 51–53]. However, we observed detrimental effects of treatment of the cell suspension of dopaminergic precursors with ROCK inhibitors, which is consistent with findings of other studies [8, 9]. The detrimental effects were observed when the dopaminergic precursors were treated before plating (i.e., before or after dissociation) and were not observed when the ROCK inhibitor was added to mature cultures (i.e., at 7 DIV). This suggests that ROCK inhibition is particularly detrimental in cells that have to migrate and become attached, as in primary cultures and grafts. Consistent with this, we did not find the detrimental effect of ROCK inhibition in cell suspensions that were not plated. The effect of ROCK inhibitors may also depend on the developmental stage of the cellular population and on distinct lineage/tissue-specific cells [8, 11], and may be more effective on iPS and ES cells than on the dopaminergic precursors.

In PD models, recent studies carried out in our laboratory [21, 26–28] and others [20, 34] have shown that ROCK inhibitors protect against dopaminergic neuron death and axonal retraction induced by dopaminergic neurotoxins (reviewed by Labandeira-Garcia et al. [23]). In the present study, treatment of the host with ROCK inhibitors only led to a slight, nonsignificant increase in the number of surviving dopaminergic neurons. However, it is known that approximately 90% of dopaminergic neurons die within 4 days after transplantation, and the number remains more stable thereafter [54, 55]. The present results suggest that, in grafted rats, the role of neuroinflammation and other factors counteracted by ROCK inhibitors in PD models are less important than additional factors responsible for neuronal death during the transplantation process. This is consistent with previous findings showing that immunosuppression with cyclosporine had no significant effect on survival of neurons in grafts between rats of the same strain [56]. The protective effect of ROCK inhibitors related with inhibition of neuroinflammation may be more important in grafts between different rat strains, in grafts in PD patients [43, 44], and in long-term protection of the grafted neurons [57]. The present results (i.e., no significant increase in dopaminergic neuron survival after host treatment) suggest that inhibition of the host ROCK activity has only minor or no detrimental effects on survival of grafted dopaminergic cells in comparison with those induced by inhibition of ROCK activity in the cell suspension (i.e., marked decrease in dopaminergic cell survival, possibly related to decreased dopaminergic cell migration within the graft, and decreased graft vascularization).

In dopaminergic grafts, after the first few days most of the surviving dopaminergic neurons are located peripherally and close to the diffusible support from the host vasculature [58, 59], whereas more resistant nondopaminergic (i.e., TH-negative) cells survive in the center of the graft. Accumulation of dopaminergic neurons in the periphery of the graft is probably related to the increased rate of dopaminergic cell death in the center of the graft, as well as to the ability of the grafted dopaminergic neurons to migrate toward the host "trophic" and/or "tropic" substances [58, 60]. Treatment of the cell suspension with ROCK inhibitors may block the migratory ability of the dopaminergic precursors, and only those dopaminergic cells that can reach the periphery of the graft or those already located in peripheral areas would survive.

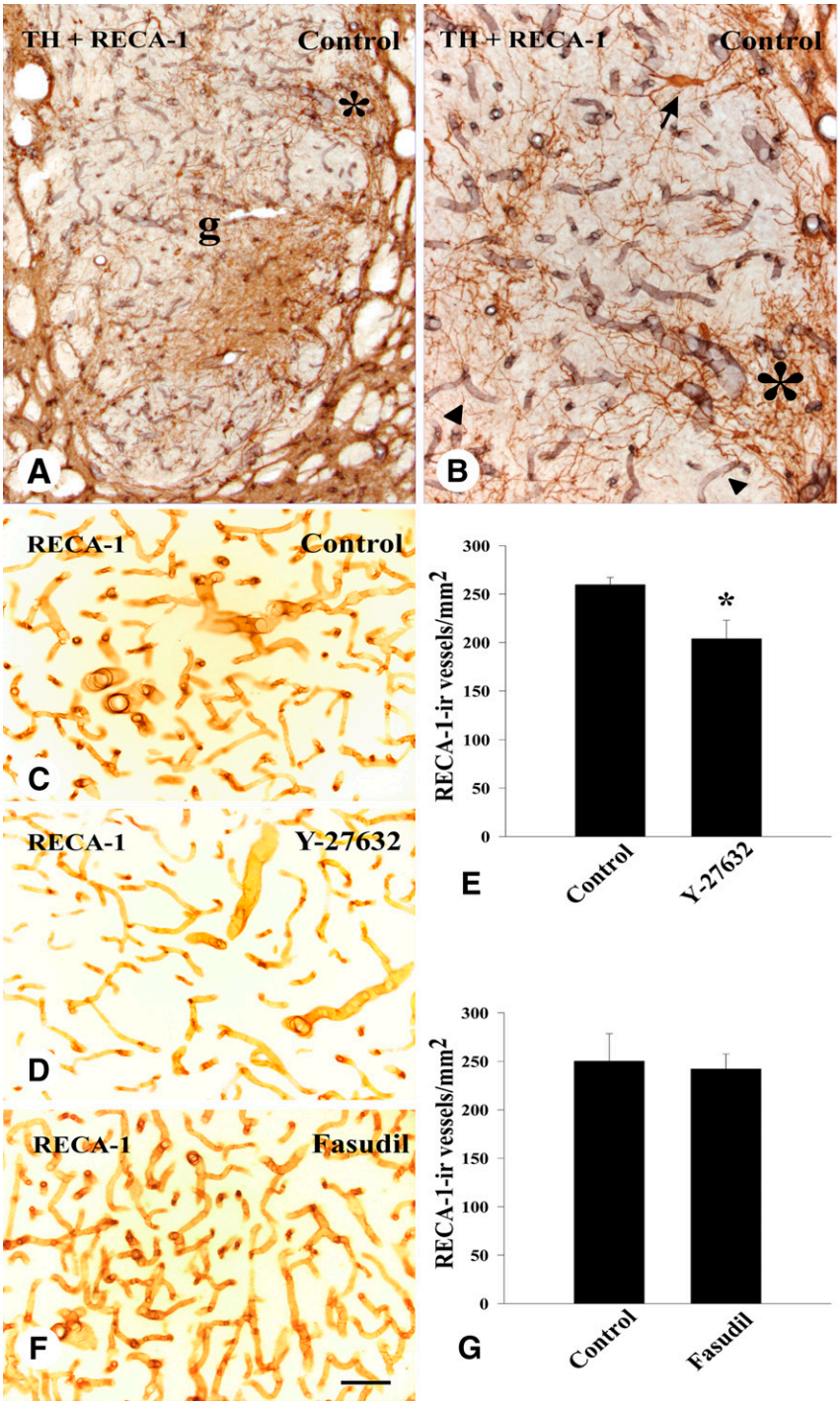

Figure 5. Density of microvessels in intrastriatal ventral mesencephalic (VM) grafts. Double immunolabeling of dopaminergic neurons (i.e., TH-ir, arrow) and microvessels (arrowheads) in VM grafts (**A**; magnified in **B**, asterisk). The density of microvessels in grafts from suspensions treated with Y-27632 **(C–E)** decreased relative to untreated controls. Treatment of host rats with fasudil did not have a significant effect on the number of RECA-1-positive vessels in the graft **(F, G)**. Scale bar = 200 μm **(A)** and 100 μm **(B–D, F)**. Data represent number of RECA-1-immunoreactive vessels per mm^2 (mean $\pm$ SEM). $*$, $p < .05$ relative to control-grafted rats (Student's *t* test). Abbreviations: g, grafts; RECA-1, rat endothelial cell antigen-1; RECA-1-ir, immunoreactive rat endothelial cell antigen-1; TH, tyrosine hydroxylase; THir, immunoreactive tyrosine hydroxylase.

ROCK Inhibition and Dopaminergic Axonal Outgrowth

In the present study, axonal outgrowth and striatal reinnervation increased significantly in host animals treated with ROCK inhibitors, which suggests that ROCK inhibition counteracts axonal collapse and retraction induced in grafted neurons by inhibitory conditions derived from the host striatum. Axon-stabilizing effects in damaged neurons have also been suggested as a mechanism of neuronal protection by ROCK inhibition [19, 61]. In neurons, ROCK activation is involved in axonal collapse and retraction in the presence of inhibitory conditions, through

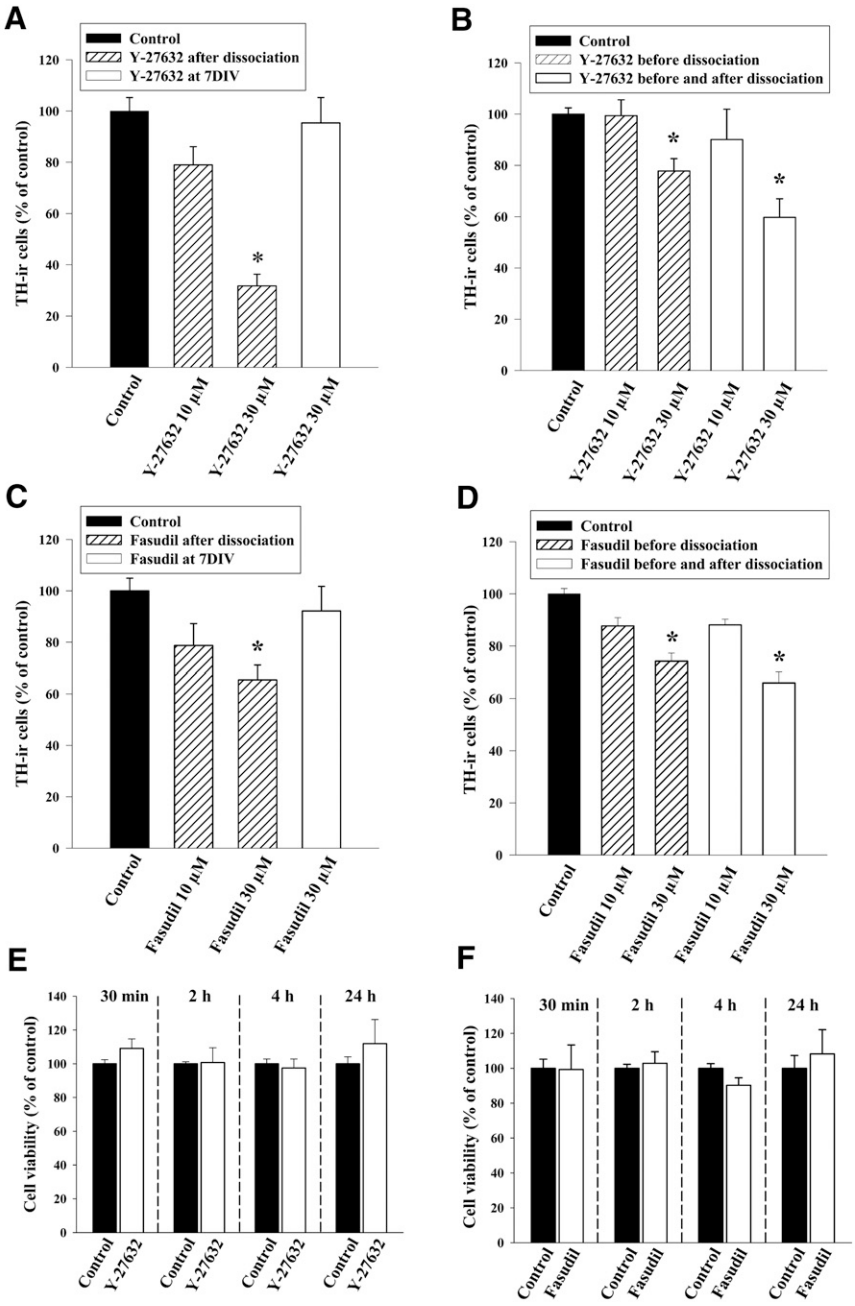

Figure 6. Effect of Rho kinase (ROCK) inhibitors on dopaminergic neuron survival in vitro. Treatment of the ventral mesencephalic cell suspensions with 30 μM Y-27632 **(A)** or 30 μM fasudil **(C)** after dissociation (i.e., at plating) induced a significant loss of dopaminergic neurons (i.e., TH-ir cells). No significant differences in the number of TH-ir cells were observed after treatment with the same dose of Y-27632 or fasudil at 7DIV relative to the control group **(A, C)**. ROCK inhibition (30 μM Y-27632 or fasudil) before or before and after dissociation also significantly reduced the number of TH-ir neurons in vitro **(B, D)**. However, lower doses of ROCK inhibitors (10 μM) did not induce a significant decrease in the survival of TH-ir cells. Cell suspensions treated (30 min–24 hours) with Y-27632 (30 μM; **D**) or fasudil (30 μM; **E**) and analyzed for cell viability before plating did not show any significant difference relative to untreated controls. Data represent mean $\pm$ SEM. $*$, $p < .05$ relative to control cultures (one-way analysis of variance followed by Bonferroni post hoc test in **A–D** and Student's *t* test in **E** and **F**). Abbreviations: 7DIV, 7 days in vitro; TH-ir, immunoreactive tyrosine hydroxylase.

modulation of myosin light chain, phosphorylation of LIM kinase and other mechanisms [61–63]. Several recent studies have shown that neuroinflammation may affect axon regeneration and growth [64, 65], and inhibition of the host neuroinflammatory response by fasudil may contribute to the observed increase in host reinnervation. However, we have recently shown that ROCK inhibitors can directly (i.e., in the absence of glia) inhibit the axonal retraction induced by neurotoxins in dopaminergic neurons [26]. In the present and previous studies [26], we observed no significant change in the number of dopaminergic neurons and neurite length in control cultures treated with ROCK inhibitors after plating. This is consistent with previous studies showing that the effect of ROCK inhibitors was minimal or completely absent under permissive conditions and that ROCK

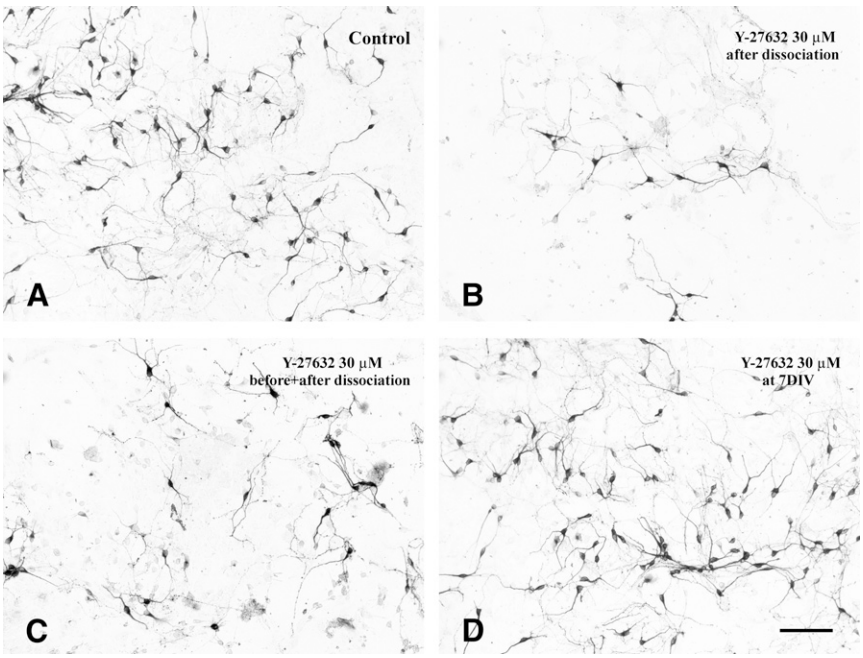

Figure 7. Microphotographs illustrating the effect of Rho kinase inhibition on dopaminergic neuron survival in vitro **(B–D)** relative to the control group (A). Treatment of ventral mesencephalic cell suspensions with 30 μM Y-27632 after dissociation **(B)** or before and after dissociation **(C)** induced a significant loss of dopaminergic neurons (i.e., TH-ir cells). However, no significant differences in the number of TH-ir cells were observed after treatment at 7DIV relative to the control group **(D)**. Scale bar = 100 μm. Abbreviation: 7DIV, 7 days in vitro.

inhibition appeared to decrease axonal retraction or stimulate growth on inhibitory substrates only (i.e., such as the host striatum), rather than causing a general effect of basal promotion of axonal extension regardless of the substrate [19, 61].

ROCK Inhibition and Graft Vascularization

Angiogenesis is largely mediated by ROCK. ROCK signaling is an important mediator in many angiogenic processes, including migration, survival, and permeability of endothelial cells [66]. In the present study, a graft vascularization decreased significantly after treatment of cell suspensions with the ROCK inhibitor Y-27632. However, no significant decrease in graft vascularization was observed after treatment of the host with fasudil. It is generally considered that vessels in dopaminergic grafts are predominantly host- and not graft-derived [67, 68]. ROCK inhibition of the cell suspension may block major graft-derived signals required to stimulate host vessels to vascularize the graft, leading to decreased graft vascularization. In addition, a recent study [69] suggests that donor-derived angiogenesis also contributes to graft vascularization. As noted earlier for migration of dopaminergic precursors, treatment of the cell suspension with ROCK inhibitors may block the migratory ability of the vascular precursors, which may lead to the decrease in graft vascularization. Future studies are necessary to clarify these mechanisms and the reason that administration of fasudil to the host is less detrimental for graft vascularization than treatment of the cell suspension with the ROCK inhibitor Y-27632. In any case, the increase in cell death observed in primary cultures (i.e., independent of vascularization) after treatment of the cell suspension with ROCK inhibitors before plating indicates that the effect on graft vascularization is not the only factor involved in the increase in dopaminergic neuron death observed after treatment of the suspension with the ROCK inhibitor.

Conclusion

The present results discourage the treatment of suspensions of dopaminergic precursors with ROCK inhibitors in the pregrafting period. However, this does not exclude possible beneficial effects on handling of stem cells in early stages of development. Treatment of the host animal with ROCK induced a marked increase in the graft-derived striatal reinnervation. ROCK inhibitors have also been shown to exert neuroprotective effects in several models of PD. Treatment of the host (i.e., PD patients) with ROCK inhibitors, currently used against vascular diseases in clinical practice, before and after grafting may be a useful adjuvant treatment to cell therapy in PD.

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Author Contributions

J.R.-P.: conception and supervision of the whole study; performance of lesion experiments, grafts, and immunohistochemistry; performance of behavioral test and data analysis; performance of cell cultures, immunohistochemical studies of cultures, and MTT assays; data analysis and interpretation; writing of the manuscript; editing of the manuscript; revision and final approval of manuscript; A.I.R.-P.: performance of lesion experiments, grafts, and immunohistochemistry; editing of the manuscript; revision and final approval of manuscript; A.M.: performance of lesion experiments, grafts, and immunohistochemistry; performance of behavioral test and data analysis; editing of the manuscript; revision and final

approval of manuscript; J.A.P.: performance of cell cultures, immunohistochemical studies of cultures, and MTT assays; editing of the manuscript; revision and final approval of manuscript; J.J.T.-A.: data analysis and interpretation, writing of the manuscript, editing of the manuscript; revision and final approval of manuscript; J.L.L.-G.: conception and supervision of the whole study, data analysis and interpretation, writing of the manuscript, editing of the manuscript, revision and final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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