

Original Article

Inhibition of the Rho signaling pathway improves neurite outgrowth and neuronal differentiation of mouse neural stem cells

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Abstract: Neurons in the adult mammalian CNS do not spontaneously regenerate axons after injury due to CNS myelin and other inhibitory factors. Previous studies have showed that inhibition of the Rho-ROCK pathway promotes axonal outgrowth in primary neurons or in spinal cord injury models. Furthermore, RhoA inhibitor C3 transferase has a potential effect to induce neural differentiation in primary cultured neurons and cell lines. As stem cells and stem cell-derived neural progenitor cells have emerged as a regenerative medicine for stroke, Parkinson's disease and other neurological disorders, strategies that can promote axonal outgrowth and neuronal differentiation appear to have promising benefits in the cell-based therapy. Currently, how changes in the Rho-ROCK pathway may affect the neurite outgrowth and neuronal differentiation of stem cells has been poorly understood. The present investigation examined the effects of RhoA inhibition on neurite outgrowth and neuronal differentiation of neural stem cells (NSCs) isolated from the subventricular zone (SVZ) of the mouse. Our results show that inhibition of RhoA leads to neurite outgrowth of NSCs not only on normal culture substrate, poly-D-lysine (PDL), but also on myelin substrate. Moreover, inhibition of RhoA improves neuronal differentiation of NSCs and up-regulates biomarkers of neuronal gene expression. These results support that the Rho signaling pathway plays an important role in neurite development and neuronal differentiation of NSCs.

Keywords: Neural stem cells, Rho signaling pathway, neurite outgrowth, neuronal differentiation, myelin

Introduction

Neural stem cells (NSCs) isolated from embryonic and adult mammalian brains and cultured as neurospheres are capable of differentiating into neurons, astrocytes, and oligodendrocytes [1, 2]. The stem cell cultures and neurally differentiated cells are excellent tools and models for studying neurogenesis and cellular mechanism for the treatment of nervous system disorders [3-5]. The grafting of NSCs improves neurological deficits in various models of neurological diseases and injuries, such as ischemic stroke, Parkinson's disease, Alzheimer's disease and spinal cord injury [6-8]. Neurogenesis is enhanced following brain injuries and new neural progenitor cells are generated in the post-injury brain [5, 9]. However, the induced new neuronal cells in the

damaged brain are very few and might be non-functional and incapable of integrating into the host brain circuitry [10, 11]. An alternative is the transplantation of neural stem cells that could differentiate into neuronal cells for tissue repair, however, transplanted cells face the same challenge of neuronal differentiation, maturation and integrating with the host neural networks [3, 4]. In this approach, sufficient neurite outgrowth and formation of correct neuronal circuits are critical steps for morphological and functional benefits of a cell replacement therapy.

Neurite outgrowth or neurogenesis and its inhibition are under balanced control during development [12]. Disruption of this control occurs in pathological states of the adult central nervous system (CNS). Neurons in the adult

mammalian CNS do not spontaneously regenerate axons after injury. CNS myelin inhibits neurite outgrowth because it contains several growth-inhibitory proteins, including myelin-associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein and Nogo as well as chondroitin sulfate proteoglycans [13-16]. The RhoA-ROCK pathway is pivotal in the control of neurite outgrowth and its inhibition. Inhibiting RhoA using C3 transferase or a dominant-negative approach promotes neurite outgrowth, even on inhibitory substrates [17]. Inhibiting ROCK with Y-27632 also promotes neurite outgrowth. Previous reports have shown that C3 transferase and Y-27632 can promote axonal regeneration in spinal cord injury and cortico-spinal tract lesion [14, 18]. RhoA activation leads to growth cone collapse and neurite inhibition in many cells and primary neurons [19]. Recent reports showed that RhoA inhibitor had the potential to induce neural differentiation [20]. However, it is currently unknown whether RhoA inhibitor can affect neurogenesis and differentiation of neural stem cells.

Materials and methods

Isolation, culture and differentiation of mouse NSCs

NSCs were isolated and cultured as previously described with some modification [21]. In brief, single cell suspensions isolated from the subventricular zone (SVZ) of neonatal brain of C57/BL6 mice were plated in proliferation medium, containing DMEM and Ham's F12 medium (DMEM/F12, 1:1) (Invitrogen, Carlsbad, CA, USA) supplement with 2% B27 (Invitrogen), 20 ng/mL epidermal growth factor (EGF) (Invitrogen) and 20 ng/mL basic fibroblast growth factor (bFGF) (Invitrogen). After 5-7 days, neurospheres were mechanically split and plated in the same medium with 1:3 ratios. In this floating system, the differentiated cells died and NSCs proliferated and formed multicellular neurospheres. Passage 2-4 NSCs were used in the present study.

For differentiation studies, neurospheres were transferred to 0.1 mg/ml poly-D-lysine coated cover slips and cultured with DMEM/F12 (1:1) supplemented with 2% B27 and 5 μ M retinoic acid (RA) (Sigma-Aldrich; St. Louis, MO, USA). The medium was changed every 2 days. After 6

days, the culture was transferred to neurobasal medium supplement with 2% B27 and 20 ng/mL nerve growth factor (NGF) (Invitrogen).

Whole-cell patch clamp recordings on NSC-derived neuronal cells

Conventional whole-cell recordings were performed in dissociated cells to record voltage-gated Na⁺ and K⁺ currents. Briefly, coverslips containing differentiated cells derived from NSCs were placed on the stage of an inverted microscope for patch clamp recordings using an EPC-9 amplifier (HEKA, Lambrecht, Germany). Recording electrodes of 6-12 M Ω were pulled from Corning Kovar Sealing #7052 glass pipettes by a Flaming-Brown micropipette puller (Corning, NY, USA). The offset potential of the tip was routinely adjusted after immersion into recording solution. Currents and traces were acquired through the PULSE/PULSEFIT program and filtered at 3 kHz by a 3-pole Bessel filter (HEKA). Recordings were performed at room temperature and pH 7.4. The extracellular solutions contained (in mM): NaCl 135, KCl 5, MgCl₂ 1, CaCl₂·2H₂O 2, HEPES 10, glucose 10. To record inward Na⁺ currents, the internal solution contained: CsCl 120, TEA-Cl 20, MgCl₂ 2, HEPES 10, EGTA 10. To record outward K⁺ currents, the internal solution contained: KCl 140, MgCl₂ 2, CaCl₂·H₂O 1, EGTA 10, HEPES 10. In the external solution, tetrodotoxin (TTX, 0.5 μ M) was added to block Na⁺ channels when K⁺ currents were recorded, and tetraethylammonium bromide (TEA, 10 mM) was added to block K⁺ channels when Na⁺ currents were recorded. All chemicals for electrophysiology studies were bought from Sigma-Aldrich (St. Louis, MO, USA).

2.3. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay

MTT assay was performed for mitochondrial and cell injury according to the manufacture instruction (Sigma-Aldrich). Briefly, neurospheres were mechanically dissociated into single cells. 3000 NSCs in 100 μ l proliferation medium were plated into 96 well plates. Permeable C3 transferase (Cytoskeleton Inc.; Denver, CO, USA) was added into the proliferation medium at different concentration and for different length of time. At the predetermined time, 10 μ l MTT reagent was added into each

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well. After 4 hrs, 10 μ l of MTT detergent reagent was added into each well and plates incubated for 2-4 hrs at 37°C in the dark. Plates were read on FL600 Microplate Fluorescence Reader (BIO TEK, Winooski, VT, USA) using a 570 nm wavelength filter.

Neurite outgrowth assay

Neurite outgrowth was performed as described previously [18, 22]. Briefly, for RhoA inhibition studies, neurospheres were dissociated and incubated with permeable C3 transferase at 1.0 μ g/ml for 4-6 hrs. Neurospheres were then collected, washed with DMEM/F12 (1:1) and plated on poly-D-lysine coated cover slips and cultured in differentiation medium. For ROCK inhibition experiments, dissociated neurospheres were incubated with 10 μ M Y-27632 (Sigma-Aldrich) or same volume of sterile H₂O in proliferation medium. For myelin inhibition experiments, cover slips were coated with 50 mg/ml poly-D-lysine and 25 μ g/ml basic myelin protein (Invitrogen) was dried down on the poly-D-lysine substrate. Slides were then coated with 10 μ g/ml laminin (Sigma-Aldrich) for 1-2 hrs. C3 or Y-27632 treated NSCs were then plated onto the myelin substrate. Neurite outgrowth quantification was performed using the ImageJ software (NIH, Bethesda, MD, USA).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (PFA) for 20 min and then permeabilized with 0.2% Triton X-100 for 10 min. Coverslips were then incubated in 5% bovine serum albumin (Sigma-Aldrich) for 1 hr before incubation with primary antibodies overnight at 4°C: nestin (MAB353; 1:200; Chemicon, Billerica, MA, USA), MAP-2 (AB5622; 1:500; Chemicon) and GFAP (AB1540; 1:100; Chemicon). Following primary antibody incubation, cells were washed with 1 \times PBS, incubated with secondary antibody Alexa Fluor 488-conjugated anti-rabbit IgG (1:200; Chemicon) or Cy3-conjugated anti-mouse IgG (1:400), washed and mounted with mounting medium with DAPI (Vector Laboratories; Burlingame, CA, USA). Staining was visualized by fluorescent microscopy (BX61; Olympus, Tokyo, Japan).

Western blot analysis

Cells were collected by centrifugation and protein isolated with RIPA buffer containing prote-

ase inhibitor cocktail (Sigma-Aldrich). Protein concentrations were determined using bicinchoninic acid assay (BCA; Pierce, Rockford, IL, USA). Proteins were then separated by SDS-PAGE gel electrophoresis, followed by transfer to PVDF membrane (Biorad, Hercules, CA, USA). Membranes were blocked for 1 hr with 5% BSA in TBST before overnight incubation with primary antibodies: ROCK-II (610623; 1:1000; BD Transduction Laboratories), Akt (sc-8312; 1:1000; Santa Cruz Biotechnol, Santa Cruz, CA), phospho-Akt (Ser473) (#9271; 1:1000; Cell Signaling Technology, Danvers, MA, USA), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (#9101; 1:1000; Cell Signaling Technology), p44/42 MAPK (Erk1/2) (137F5) (#4695, 1:1000; Cell Signaling Technology), RhoA (67B9) (#2117, 1:1000; Cell Signaling Technology), α -tubulin (sc-5286; 1:5000; Cell Signaling Technology) or β -actin (A5316, 1:5000; Sigma-Aldrich). Blots were washed and antigen binding was detected by incubation with anti-rabbit or anti-mouse HRP-conjugated secondary antibodies (Bio-Rad; Hercules, CA, USA) and detection by the ECL method (Thermo Scientific; Hudson, NH, USA). Chemiluminescence was detected on CL-XPosure radiographic films (Thermo Scientific) and intensity was analyzed by the ImageJ software (NIH). To compare relative protein intensity across groups, loading was normalized to β -actin expression.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen) and purified with QIAGEN RNeasy mini Kit (QIAGEN, Turnberry Lane Valencia, CA, USA). The concentration of total RNA was measured with Nanodrop 1000 (Thermo Fisher Scientific). Semi-quantitative RT-PCR was performed using the QIAGEN OneStep RT-PCR Kit (QIAGEN) following the manufacturer's protocols. 500 ng or 1 μ g mRNA of each sample was used for PCR reaction. The following conditions were used: 94°C 30 sec, 58-62°C 30 min, 72°C 1 min, 25-30 thermal cycles. The PCR products were visualized with 1.5% agarose gel. mRNA levels were normalized to mouse house-keeping gene 18S rRNA. The primer sequences used are listed in **Table 1**.

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Table 1. Primers for RT-PCR analysis

| Primer | Forward Primer | Reverse Primer |
|----------------------------|--------------------------|------------------------|
| Neurofilament-L (NFL) | CCGTACTTTTCGACCTCCTACA | CTTGTGTGCGGATAGACTTGAG |
| Doublecortin (DCX) | AAACTGGAACCGGAGTTGTC | CGTCTTGGTCGTACCTGAGT |
| MAP-2 | ACATCAAACATTCTGCTGGGGGCG | CAAGCGCCGCAGTGACATCCT |
| GFAP | CGGAGACGCATCACCTCTG | AGGGAGTGGAGGAGTCATTCTG |
| Myelin basic protein (MBP) | CCCAAAGAATAACTGGCAAGGC | GAAGCTCGTCGGACTCTGAG |
| Proteolipid protein (PLP) | TGAGCGCAACGGTAACAGG | GGGAGAACACCATACATTCTGG |
| 18S rRNA | TCGCTCGCTCCTCTCCTACTTG | CCTGCTGCCTTCCTTGGATGTG |

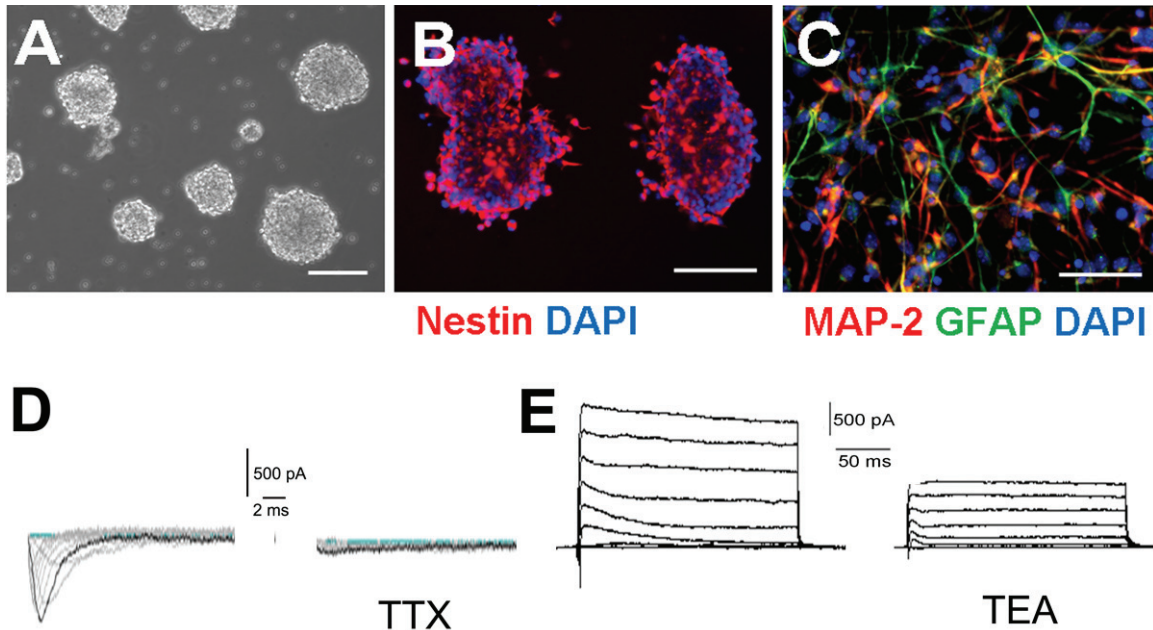


Figure 1. Cell culture and characterization of NSCs from mouse SVZ. A. Cells isolated from mouse brain SVZ region formed neurospheres after 7 days in culture. B. Many cells in neurospheres were neural progenitor marker Nestin (red) positive. DAPI (blue) staining shows all cells. C. 12 days after neuronal differentiation with the RA protocol, many cells became positive to the neuronal marker MAP-2 (red) or astrocyte marker GFAP (green). Scale bar = 100 μ m. D. Voltage-gated Na⁺ currents were recorded in neuron-like cells 12 days after differentiation. The inward current was triggered by voltage steps from -70 mV to +50 mV in 10 mV increments in the presence of K⁺ channel blockers. Individual current traces are superimposed in the figure. Bath application of the selective Na⁺ channel blocker tetrodotoxin (TTX, 500 nM) completely blocked the voltage-gated inward current. E. Voltage-gated outward K⁺ currents were recorded in the presence of TTX. Individual current traces evoked by depolarizing voltage steps are superimposed in the figure. The outward currents were inhibited by the K⁺ channel blocker 3 mM TEA. Traces are representative for recordings from more than 15 cells.

Statistical analysis

Data were graphed and analyzed using either Origin (OriginLab Corporation, Northampton, MA, USA) or SigmaPlot (SyStat Software, San Jose, CA, USA) software. Student's two-tailed t-test was used for comparison of two experimental groups. Multiple comparisons were performed using one-way ANOVA followed by Tukey post-test. Significance was deter-

mined based on standard error of mean and defined as $P < 0.05$.

Results

Characterization of NSCs isolated from SVZ of the mouse brain

The neural stem cell marker Nestin was used to detect NSCs by immunocytochemical staining.

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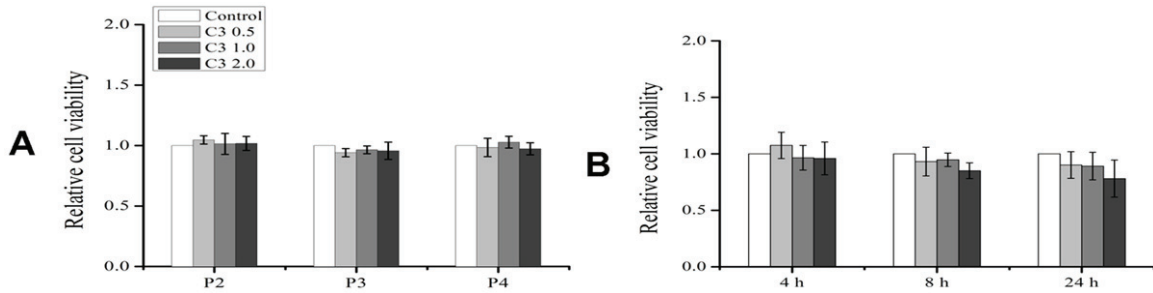


Figure 2. Effect of C3 phosphatase on cell viability of NSCs. Cell viability after C3 phosphatase treatment was measured using MTT assay. A. NSCs of passage 2, 3 and 4 (P2, P3 and P4) were exposed to different concentration (0.5, 1.0 and 2.0 µg/ml) of C3 phosphatase for 4 hrs, MTT assay was performed 24 hrs later. No change in cell viability was detected in all tests. B. NSCs were subjected to different duration of 1.0 µg/ml C3 treatment and MTT assay was performed 24 hr later. Prolonged exposure to C3 phosphatase for 24 hrs resulted in about 20% reduction in cell viability. N ≥ 3 independent assays per group. P > 0.05 for all comparisons between experimental groups.

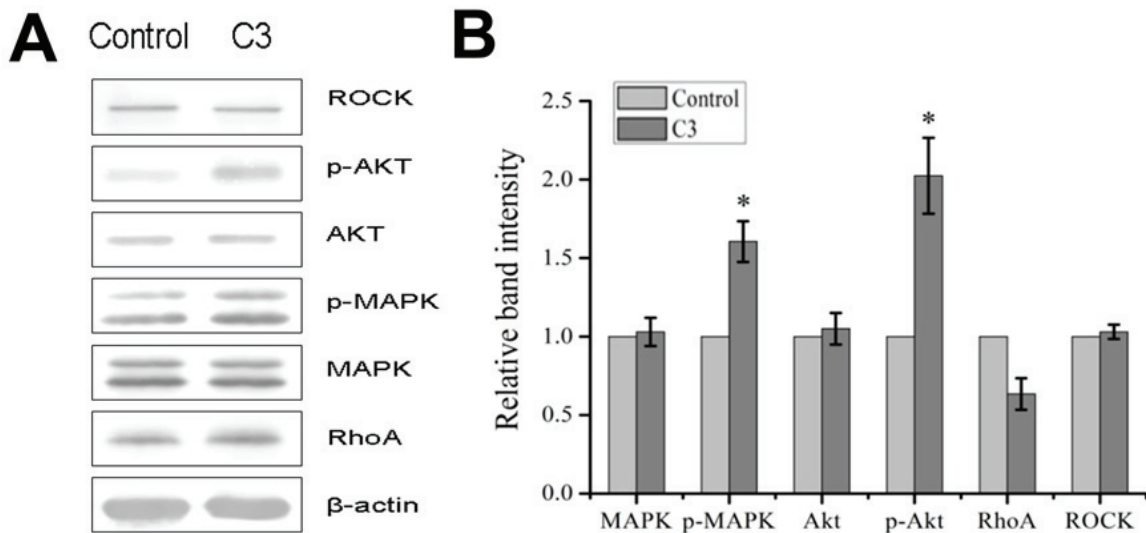


Figure 3. Regulation of MAPK pathway molecules in C3-treated NSCs. Western blot was applied to measure the protein levels of signaling molecules in the MAPK pathway. A. Representative immunoblots with antibodies against MAPK, pMAPK, Akt, pAkt, ROCK II and RhoA in cells exposed to 4-hr C3 (1.0 µg/ml) treatment. B. Quantification of Western blot bands for MAPK/pMAPK, Akt/pAkt, ROCK II and RhoA in C3 (1.0 µg/ml) treated cells. *, p < 0.05 compared with control group. N = 4 assays.

Differentiated neuronal cells were identified using the neuronal-specific marker microtubule-associated protein-2 (MAP-2). Glial differentiation was revealed by GFAP immunofluorescence. After seven days in culture, NSCs formed neurospheres showed Nestin positive immunoreactivity (Figure 1A and 1B). Retinoic acid (RA), a derivative of vitamin A and a developmental morphogen, has been widely used for neural induction of pluripotent stem cells [23]. During 12-16 day RA induction in NSC cultures, NSCs differentiated into neural progenitor cell lineage and became neuronal and glial like

cells (Figure 1C). Whole-cell voltage clamp recording verified that neuronal specific Na⁺ current could be detected in neuron-like cells after 12-16 day differentiation. Meanwhile, fast-inactivated and non-inactivated outward K⁺ currents that are essential for normal neuronal activities were also observed in these cells (Figure 1D-G).

The effects of C3 transferase on cell viability of NSCs

ROCK inhibitors were shown to increase survival of dissociated human embryonic stem

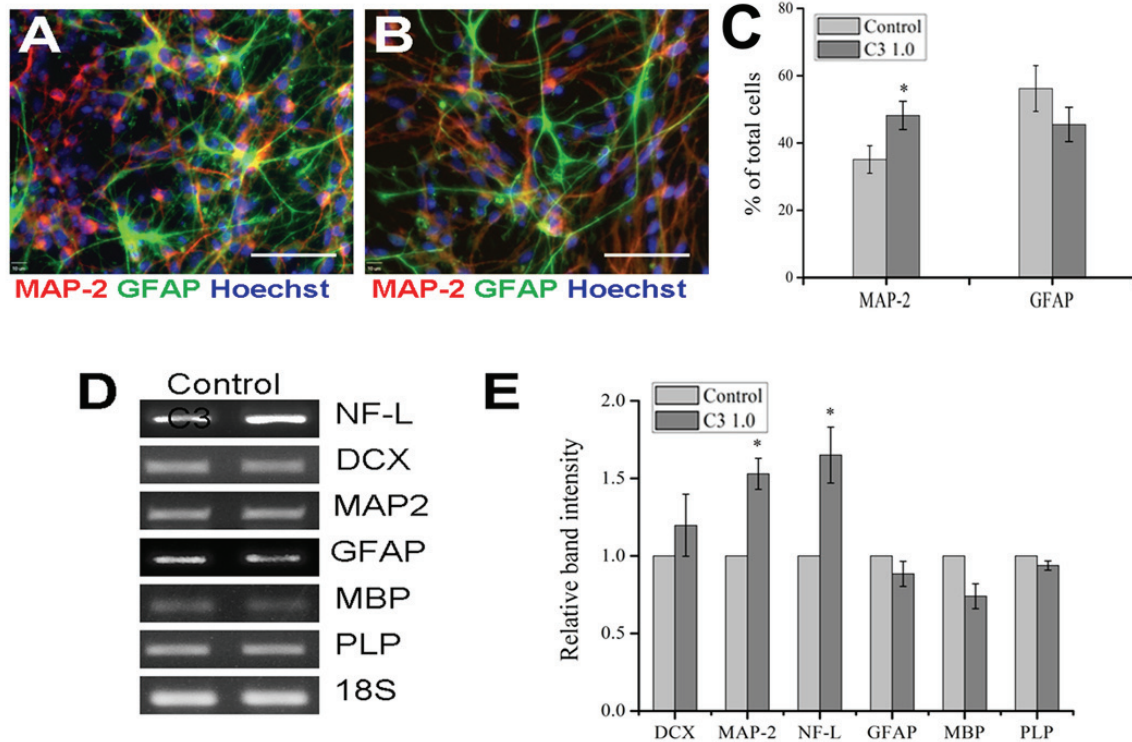


Figure 4. Neuronal differentiation of NSCs after C3 pretreatment. C3 treatment promoted neuronal differentiation of NSCs. A and B. MAP-2 (red) and GFAP (green) fluorescent images of control cells (A) and C3 phosphatase treated cells (B). C3 (1.0 $\mu\text{g}/\text{ml}$, 4 hrs) pretreatment was performed before the 12-16 day neuronal differentiation using the RA protocol. C. Percentage quantification of NeuN-positive and GFAP-positive cells 12-16 days into neuronal differentiation. *. $p < 0.05$ compared with control; $n \geq 3$ assays. C3 pretreatment significantly increased NeuN-positive cells compared to control cells. There is a trend of reduction in GFAP-positive cells in C3-treated group. D and E. Gene expression changes after 1.0 $\mu\text{g}/\text{ml}$ C3 treatment detected using RT-PCR. Quantified analysis is shown in E. MAP-2 and NF-L expression was significantly increased in C3-treated cells. *. $p < 0.05$ vs. control group; $n = 3$ assays.

cells [24]. To delineate whether blocking the Rho pathway might affect viability of NSCs, MTT assay was performed in the absence and presence of the cell permeable Rho inhibitor C3 transferase. C3 transferase incubation at 0.5 to 2.0 $\mu\text{g}/\text{ml}$ for 4 hrs did not affect the viability of NSCs at passages 2 to 4 (Figure 2A). Longer incubation treatments with 2 $\mu\text{g}/\text{ml}$ of C3 transferase for 24 hrs showed only a trend of reduction in cell viability (Figure 2B). Based on these results NSCs were subsequently treated with 1 - 2 $\mu\text{g}/\text{ml}$ of C3 transferase for no longer than 4-6 hrs in the following experiments.

C3 transferase increases expression of p-MAPK and p-Akt in NSCs

Western blot was performed 4 hrs after 2 $\mu\text{g}/\text{ml}$ C3 treatment to evaluate the changes of two

important signaling pathway effectors, phosph-Akt and phosph-MAPK. Treatment with C3 transferase significantly increased phosph-Akt and phosph-MAPK in NSCs with no effect on MAPK or Akt expression levels (Figure 3A and 3B).

C3 transferase pretreatment improves neuronal differentiation of NSCs

C3 transferase may act as an exogenous factor to stimulate differentiation of primary neuronal cultures [25, 26]. NSC neuronal differentiation was induced by the RA protocol described in Methods. After 12-16 hrs into neuronal differentiation, MAP-2 and GFAP staining exhibited increased MAP-2-positive cells and a tendency of reduced GFAP-positive cells (Figure 4A-C). RT-PCR results showed that C3 transferase pretreatment (2 $\mu\text{g}/\text{ml}$, 4 hr exposure before

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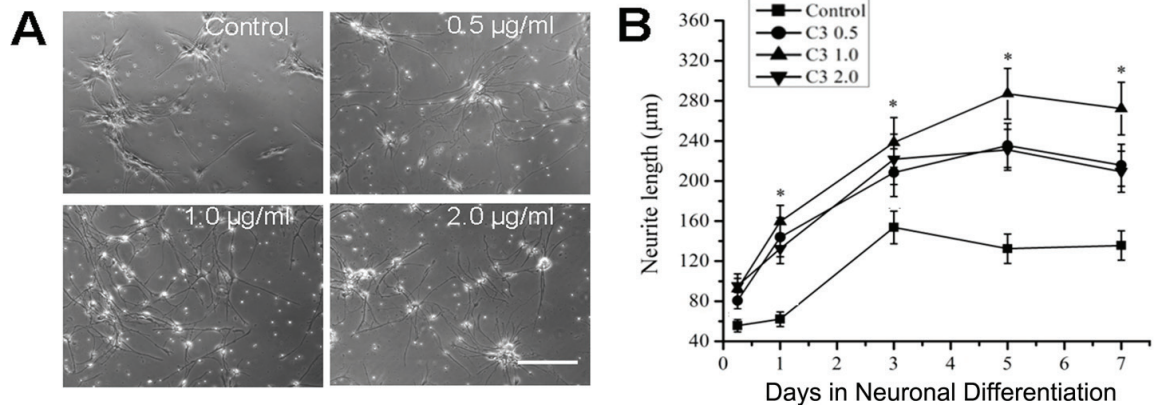


Figure 5. C3 transferase promotes neurite outgrowth of NSCs. NSC cultures was pre-treated with C3 for 4 hrs for its effect on neurite development A. Phase contrast images were taken 5 days after plating from NSCs pre-treated with different C3 concentrations. Scale bar: 200 µm. B. Quantification of neurite length of NSCs at different time points. *. $p < 0.05$ compared with time matched controls. $N \geq 5$ independent assays.

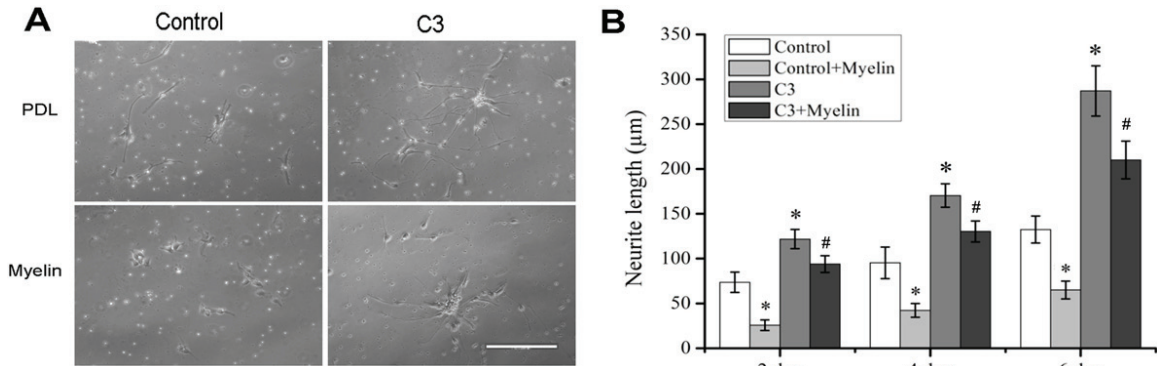


Figure 6. C3 transferase promotes neurite outgrowth of NSCs on myelin substrate. NSCs were planted on myelin substrate for the effect of C3 pre-treatment on neurite development. A. Phase contrast photos were taken 2 days after planting from NSC cultures treated with C3 (1.0 µg/ml). B. Quantification of neurite length of NSCs under different conditions and at different time after neuronal differentiation. *. $p < 0.05$ compared with poly-D-lysine coated controls, #. $p < 0.05$ compared with myelin control. Scale Bar=200 µm.

RA induction) upregulated neuronal gene expression in NSCs (Figure 4D and 4E). MAP-2 and NF-L, two markers of mature neurons, showed significant increases in C3-treated NSC cultures compared to untreated controls that underwent the same RA protocol (Figure 4C-E). Markers of astrocytes (GFAP) and oligodendrocytes (MBP and PLP) were not significantly changed following C3 treatment (Figure 4C-E).

C3 transferase pretreatment increases neurite outgrowth of NSCs

Rho GTPase and its effectors play a critical role in regulating actin dynamics and Rho-ROCK signaling pathway contributes to diverse neuronal functions from neuritogenesis to cell survival and neuronal differentiation [16]. Pretreatment

of NSCs for 4 hrs with C3 transferase dramatically increased neurite outgrowth during the consequent period of neuronal differentiation. The effect of C3 was concentration dependent with 1 µg/ml being most effective (Figure 5A-B). The effect of C3 was visible at day 1 post differentiation with a mean neurite length of 150 ± 20 µm for the C3 group compared to 63 ± 6 µm in control group ($p < 0.05$, $n = 5$). After 5 days in culture, neurites of C3-treated NSCs were approximately twice as long as the non-treated groups (Figure 5B).

C3 transferase pretreatment promotes NSCs neurite outgrowth on myelin substrate

Myelin is one of the components of the glial scar that develops following brain injury. Neurite

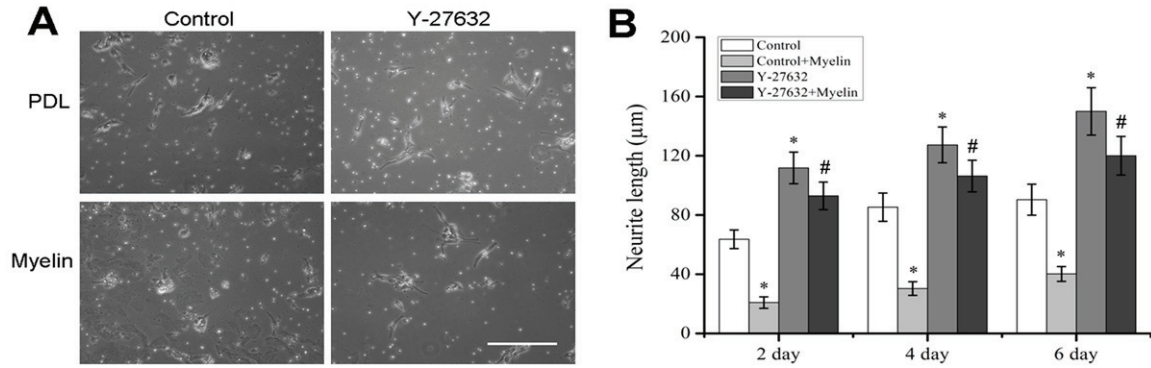


Figure 7. Y-27632 promotes neurite outgrowth of NSCs on myelin substrate. The effect of ROCK inhibitor Y-27632 on neurite outgrowth of NSCs was compared on poly-D-lysine (PDL) coated and myelin coated culture dishes. Y-27632 (10 μ M, 4 hrs) was applied before the RA induction. A. Images of NSCs cultures on PDL or myelin base under control and Y-27632 treated condition. B. Quantification of neurite length of NSCs and comparisons between different culture conditions. *. $p < 0.05$ compared with PDL control group, #. $p < 0.05$ compared with myelin control. $N \geq 5$ assays.

outgrowth of NSCs was significantly reduced when NSCs were cultured on myelin substrate, compared to NSCs cultured on PLL substrate (Figure 6). When NSCs were pretreated with 1 μ g/ml of C3 transferase prior to plating, the myelin-induced inhibition diminished and neurite length was significantly longer compared to the myelin control condition (Figure 6B). Six days post plating the neurite length of C3-pretreated cells was 3 times longer than those of control cells on myelin substrate, although it was still about 30% shorter than that in C3-treated cells grown on PLL (Figure 6B).

ROCK is a downstream effector protein of the small GTPase RhoA and is one of the major regulators of the cytoskeleton. ROCK activation reduced neurite outgrowth in primary neurons. We used the ROCK inhibitor Y-27632 to confirm whether C3 transferase treatment increased neurite outgrowth of NSCs through Rho-ROCK pathway. As expected, Y-27632 (10 μ M, 4 hrs) pre-treatment increased neurite outgrowth of NSCs, even on myelin substrate, although the effect was not as dramatically as C3-transferase (Figure 7).

Discussion

Although previous investigations showed that inhibition of the Rho-ROCK pathway promotes axonal outgrowth in primary neurons or in spinal cord injury models [16, 18], information on the regulation of this signaling pathway in neu-

ronal differentiation and neurite outgrowth of stem cell-derived neurons is limited. Our investigation examined the hypothesis that RhoA inhibition could significantly promote neuronal differentiation and neurite outgrowth in NSCs isolated from the SVZ of the mouse. We show that inhibition of RhoA improves neuronal differentiation of NSCs and up-regulates biomarkers of neuronal gene expression. Interestingly, inhibition of RhoA not only leads to neurite outgrowth of NSCs in normal culture substrate PDL, but also on myelin substrate. These observations provide a new approach to improve stem cell culture systems for in vitro research on drug development and disease model investigations. The results may also imply new strategies to enhance neuronal differentiation in stem cell therapy and enhance axonal growth in the post-injury microenvironment where axons exhibit very poor regeneration ability due to increased inhibitory cues such as myelin.

Myelin is thought to be one of the major inhibitors for axon regeneration in the CNS. CNS myelin is produced by oligodendrocytes and released from damaged nerve fibers [27]. Amongst CNS myelin, Nogo-A is predominantly expressed in the CNS [28]. Nogo binds its receptor NgR, activates RhoA and ROCK. The Rho/ROCK pathway has been mainly identified to mediate myelin action and inhibit neurite elongation [29]. Rho and ROCK regulate phosphatidylinositol-3-kinase/protein kinase Akt, the insulin receptor substrate-1 and various cytoskeleton organization regulating proteins

[14, 15]. Activation of the Rho-ROCK pathway inhibits axonal regeneration in many cell lines while inhibiting Rho-ROCK pathway promotes axonal outgrowth *in vitro* and *in vivo* [18, 22]. Our observation demonstrates similar molecule mechanism of neurite outgrowth of NSCs. Previous studies have shown that inhibition of the Rho-ROCK pathway regulates cell survival, proliferation and regeneration through intrinsic signaling pathway, such as MAPK and Akt signaling pathways [30]. Consistently, we observed increased phosphor-Akt and MAPK in C3-treated NSCs, suggesting a regulatory role of these molecules in the process.

In addition to reports that the Rho-ROCK pathway is associated with neural differentiation [25, 26], our previous studies showed that ERK 1/2 phosphorylation is a key event required for early neuronal differentiation and survival of embryonic stem cells [31]. In the present study, we noticed that neuronal differentiation of C3-treated NSCs was enhanced compared to that of non-treated NSCs. We demonstrate that Akt and ERK pathways may involve in the effects of RhoA on neurite growth and neuronal differentiation of NSCs. Interestingly, RT-PCR results showed that while C3 up-regulated neuronal biomarkers, it down-regulated non-neuronal biomarkers in NSCs. This is consistent with an increased neuronal differentiation and some previous reports [22, 25].

The promoting effects on neuronal differentiation and neurite growth of NSCs were observed after termination of 4 hr exposure to the RhoA inhibitor C3 phosphorase. This protocol suggests that NSCs can be primed before subjection to neuronal differentiation. It may be possible that the *in vitro* pretreatment with a RhoA inhibitor may be applied in stem cell transplantation therapy using NSCs and other stem cells to promote their neuronal differentiation and axonal growth after transplantation into the damaged CNS.

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