

Regulation of Dendritic Spine Morphology by the Rho Family of Small GTPases: Antagonistic Roles of Rac and Rho

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Dendritic spines mediate most excitatory transmission in the mammalian CNS and have been traditionally considered stable structures. Following the suggestion that spines may 'twitch', it has been recently shown that spines are capable of rapid morphological rearrangements. Because of the role of the small GTPases from the Rho family in controlling neuronal morphogenesis, we investigated the effects of several members of this biochemical signaling pathway in the maintenance of the morphology of extant dendritic spines by combining biolistic transfection of pyramidal neurons in cultured cortical and hippocampal slices with two-photon microscopy. We find a variety of effects on the density and morphology of dendritic spines by expressing either constitutively active or dominant negative forms of several small GTPases of the Rho family, by blocking the entire pathway with *Clostridium difficile* toxin B or by blocking Rho with C3 transferase. We propose a model where Rac promotes spine formation, while Rho prevents it. We conclude that the small GTPases provide antagonistic control mechanisms of spine maintenance in pyramidal neurons.

Introduction

Dendritic spines were first described by Cajal, who pointed out that cerebellar Purkinje cell dendrites had small thorns that projected from them like leaves from a tree (Ramón y Cajal, 1888). In 1891 he put spines in the spotlight of the function of the nervous system by proposing that they serve to connect axons and dendrites (Ramón y Cajal, 1891). In the 1950s, the introduction of electron microscopy enabled investigators to explore the structure of dendritic spines with unprecedented detail. These studies confirmed Cajal's neuronal theory and his prediction that spines were the site of synaptic contacts (DeRobertis and Bennett, 1955; Palay, 1956; Gray, 1959).

Traditionally, spines have been assumed to be relatively stable structures and, as a consequence of this belief, most ultrastructural work has relied on the classification of spines according to their morphology. This idea was challenged in 1982, when Francis Crick proposed that spines could move ('twitch') in response to synaptic stimulation and that this motility was actin based (Crick, 1982). A similar idea had been proposed in a previous study from Siekevitz's laboratory, in which actin was identified at the postsynaptic density using both biochemical and immunochemical methods (Blomberg *et al.*, 1977). Confirming, at least in part, these suggestions, it has recently been shown that spines in dissociated cultures (Fischer *et al.*, 1998) and in brain slices (Dunaevsky *et al.*, 1999) are capable of rapid motility. Thus, spine morphology is dynamic on a time scale of seconds and significant changes in shape occur even among relatively mature spines in animals well past the developmental period of dendritogenesis [postnatal day (P) 20+ mice] (Dunaevsky *et al.*, 1999). These novel data have also shown that spine motility is intrinsic to the neuron and is controlled by actin. It therefore has become important to understand in detail

which pathways control actin polymerization and depolymerization in spines because these pathways could be responsible for the continuous remodeling of the morphology of dendritic spines.

The role of small GTPases in controlling actin cytoskeletal reorganization and cell morphology is well established (Hall, 1994). In the CNS, GTPases from the Rho family (Rho, Rac and Cdc42) have been shown to be expressed in hippocampal pyramidal neurons and dentate granule cells, the neocortex and other regions (Olenik *et al.*, 1997). In addition, small GTPases are likely to be distributed in dendrites of pyramidal neurons, since the effector of Rho, Rho-kinase, exists in the dendrites (Hashimoto *et al.*, 1999). Rho GTPases have been shown to play an important role in the dendritic remodeling of *Xenopus* retinal ganglion cells (Ruchhoeft *et al.*, 1999) and of cultured cortical neurons (Threadgill *et al.*, 1997). Also, in transgenic mice expressing constitutively active human Rac1, the dendritic spines of Purkinje cells were increased in number and reduced in size (Luo *et al.*, 1996). These data suggest that small GTPases from the Rho family regulate dendro- and spinogenesis, although it is not yet known if they are involved in the maintenance of extant dendritic spines. To investigate this issue, and to better understand the cellular mechanisms responsible for the maintenance of the morphology of spines, we have manipulated the expression of several members of the Rho network in cortical and hippocampal pyramidal neurons in cultured brain slices from the mouse. Combining two-photon microscopy and biolistic co-transfection with enhanced green fluorescent protein (EGFP) and dominant negative or constitutively active variants of several members of this pathway, we have detected a variety of morphological rearrangements in spines and dendrites from pyramidal neurons. Based on our data we propose a model for the effect of this pathway on spine morphology.

Materials and Methods

Slice Cultures

All experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH publication no. 86-23, revised 1987). Cortical and hippocampal brain slices were prepared from postnatal day P0-P3 C57BL mice, using a tissue chopper in sterile conditions. Slices were mounted on 0.4 mm culture inserts (Millipore, Bedford, MA, PICM ORG 50) and incubated (5% CO₂, 37°C) with culture medium, composed of 50 ml basal Eagle's medium (Gibco, Gaithersburg, MD), 25 ml Hank's balanced salt solution (Gibco), 25 ml horse serum (Hyclone, Logan, UT), 0.65 g dextrose, 1.0 ml HEPES (Gibco) and 1.0 ml 100X Pen-strep (Gibco).

Plasmids and Reagents

Rac1N17 and RhoAV14 were expressed in the pEXV vector (Ridley and Hall, 1992; Ridley *et al.*, 1992) and were myc-tagged. Rac1V12 (Ridley *et al.*, 1992) was subcloned into pSRa (Takabe *et al.*, 1988) at the *EcoRI* site. Cdc42HsV12 and Cdc42HsN17 (Kozma *et al.*, 1995) were expressed

using the pCMV expression vector and were also myc-tagged. C3 transferase was expressed using the RcCMV vector. All genes were cloned from human libraries. Finally, *Clostridium difficile* toxin B was obtained from Drs Hoffman and Aktories (Freiburg, Germany) and was dissolved to 30–150 ng/ml from stocks at an initial concentration of 500 ng/μl in 50 mM Tris-HCl (pH 7.5), 500 mM NaCl and 20% glycerol.

Particle-mediated Gene Transfer

Cortical and hippocampal slice cultures were transfected using the Helios Gene Gun System (Bio-Rad, Hercules, CA). Plasmids were purified on a QIAGEN (Valencia, CA) column and precipitated onto gold microcarrier particles (1 μm diameter) according to the Helios Gene Gun instruction manual. For co-transfection, the DNA loading ratio was 8 μg/mg gold for each construct. For control experiments with EGFP, the ratio was also 8 μg/mg gold. Cultured slices were transfected at 6–19 days *in vitro* (DIV). Images were taken 2–4 days after transfection.

Two-photon Microscopy

Imaging was carried out with a custom-made two-photon laser scanning microscope consisting of a modified Fluoview (Olympus, Melville, NY) confocal microscope and a Ti:sapphire laser providing 130 fs pulses at 75 MHz at wavelengths of 740–850 nm (Mira, Coherent, Santa Clara, CA) pumped by a solid-state source (Verdi, Coherent). A ×40, 0.8 NA water immersion objective (IR1, Olympus) was used. Fluorescence was detected using photomultiplier tubes (HC125-02, Hamamatsu, Japan) in external, whole-area detection mode, and images were acquired using Fluoview (Olympus) software. Images of spines were acquired at the highest digital zoom (×10), resulting in a nominal spatial resolution of 20 pixels/μm. Pyramidal neurons were selected from CA1–3 in the hippocampus or extragranular layers of the primary visual cortex. Spines were chosen randomly from all areas of neurons, including basal, apical and oblique dendrites. For each image, several (6–15) focal planes ~1 μm apart were scanned; these were later projected into a single image. Image processing and analysis was done with custom-written macros using NIH-Image and Macintosh computers. Measurements are reported as mean ± SEM.

Immunocytochemistry

Imaged slices were fixed with 4% paraformaldehyde overnight. Slices were incubated in 1% Triton X in phosphate-buffered saline (PBS) for 30 min and blocked by 20% bovine serum albumin in PBS. Slices were then incubated with primary antibody for 4 h (anti-myc IgG, Covance, Richmond, CA) and then with secondary antibody for 4 h (Cy3-conjugated anti-mouse IgG, Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Images were taken with a Olympus BX50WI microscope and SIT camera (C2400, Hamamatsu).

Results

The Rho Signaling Network

Members of the Rho family of small GTPases comprise a subgroup of the Ras superfamily of GTPases (Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). These proteins function as molecular switches that cycle between an inactive GDP-bound form and an active GTP-bound form. Activation of the GTPases is catalyzed by guanine nucleotide exchange factors (GEFs) which exchange GDP for GTP in response to various signals. Inactivation is catalyzed by GTPase-activating proteins (GAPs) and nucleotide dissociation inhibitors (GDIs). GAPs catalyze the hydrolysis of the bound GTP, while GDIs sequester the GTPases in the cytosol and prevent nucleotide exchange and interaction with GEFs.

The Rho family includes at least 14 members. A summary of the Rho pathway is outlined in Figure 1. The better characterized of these proteins are Cdc42, Rac1 and 2, and RhoA, B and C. These GTPases have been implicated in a diverse range of processes, including regulation of cell morphology, activation of

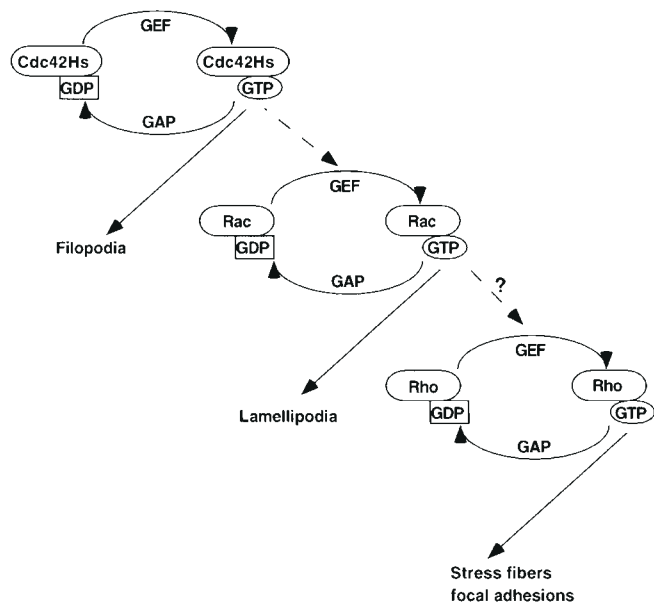


Figure 1. Summary model for the Rho family GTP signaling network. This proposed model is based on data from fibroblasts and epithelial cells. The stimulation of Rho by activated Rac is controversial.

signal transduction pathways, and regulation of cell proliferation and differentiation (Bagrodia *et al.*, 1995; Coso *et al.*, 1995; Minden *et al.*, 1995; Zhang *et al.*, 1995; Brown *et al.*, 1996; Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). The Rho family members were originally identified as proteins that regulate the organization of the actin cytoskeleton in fibroblasts. Microinjection of Cdc42Hs into fibroblasts and a variety of other cell types causes the transient induction of filopodia – thin, actin-rich protrusions at the cell surface. This is followed by the formation of thin sheets of actin at the cell surface known as membrane ruffles or lamellipodia. While the induction of filopodia is caused by Cdc42Hs activation, the induction of lamellipodia is probably due to the ability of Cdc42Hs to activate Rac. Thus, co-expression of Cdc42Hs with a dominant negative Rac mutant results in the sustained induction of filopodia without the subsequent induction of lamellipodia. Consistent with this, microinjection of activated Rac leads to the induction of lamellipodia, but not filopodia (Kozma *et al.*, 1995; Nobes and Hall, 1995). Microinjection of a third GTPase, RhoA, into fibroblasts, leads to an increased formation of actin bundles that traverse the cell, known as stress fibers, as well as focal adhesions, where cells attach to the extracellular matrix. (Ridley and Hall, 1992). Cdc42, Rac and RhoA were originally proposed to act in a linear cascade, whereby Cdc42 activates Rac, which in turn activates RhoA (Nobes and Hall, 1995). Recent work, however, has suggested that, rather than activating Rho, in some cells Cdc42Hs and Rac may actually antagonize the effects of Rho, thus leading to a reduction in stress fiber formation and contractility (Dutartre *et al.*, 1996; Manser *et al.*, 1997; Burridge, 1999; van Leeuwen *et al.*, 1999).

GFP Transfection Does Not Alter Dendritic or Spine Morphology

For most of our experiments we co-transfected neurons with two constructs: an EGFP driven by a CMV promoter, and a second construct consisting of a small GTPase in a mammalian

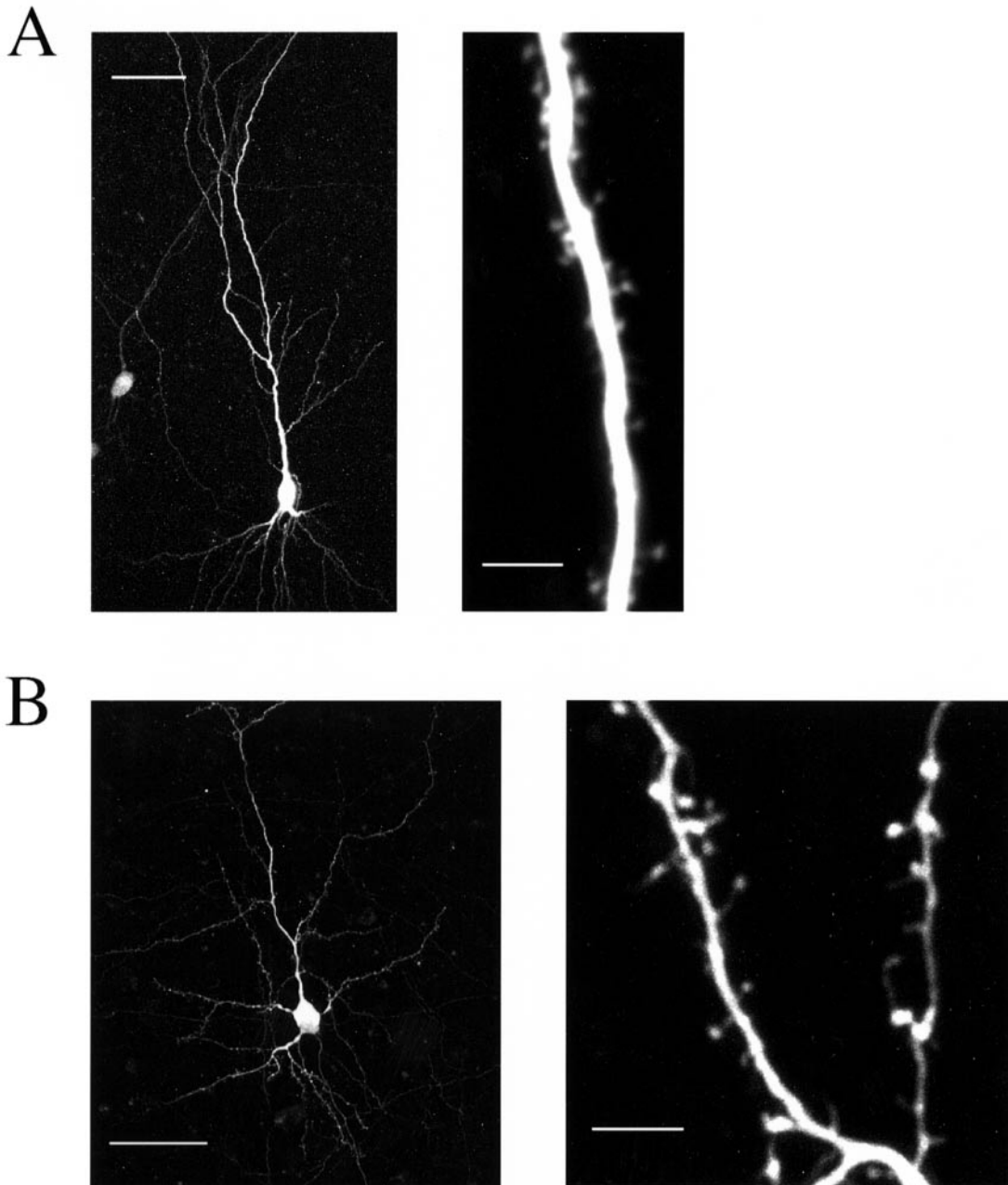


Figure 2. EGFP transfection does not alter neuronal or spine morphology. Representative low (left) and high (right) magnification two-photon images of GFP-transfected pyramidal neurons from hippocampal (*A*) and cortical (*B*) slice cultures. Spines appeared normal in both their densities and morphologies. Scale bars: 50 μm in low magnification, 5 μm in high magnification. Neuron A was a hippocampal pyramidal cell transfected at 7 DIV and imaged at 11 DIV. Neuron B was a cortical pyramidal cell transfected at 7 DIV and imaged at 10 DIV.

expression vector. The use of EGFP enabled us to image with detail the morphology of the dendrites and spines of transfected neurons, while at the same time manipulating individual members of the cascade with the second construct.

To ensure that the EGFP transfection was not affecting dendritic or spine morphology, we first examined neurons transfected only with EGFP. In each slice, dozens of cells were fluorescently labeled 2 days after transfection. After 4 days, the intensity of fluorescence decreased, although the time course of EGFP expression varied from cell to cell, even among neurons of the same cell type. EGFP-labeled cells could be classified as

pyramidal and non-pyramidal. Hippocampal and cortical pyramidal cells had a long apical dendrite and a skirt of basal dendrites (Figure 2) (Feldman, 1984). Dendritic trees were studded with spines, at average densities of 0.7 ± 0.07 spines/ μm ($n = 226$ spines from six cells). Spines ranged in length from 0.5 to 3 μm (average = 1.3 ± 0.08 μm , $n = 226$ spines from six cells) and from 0.5 to 2 μm in diameter. Structures smaller than 0.5 μm might not be detected by our microscope, and thus these measurements underestimate their true densities. We therefore concluded that EGFP transfection did not alter the morphology of the neurons.

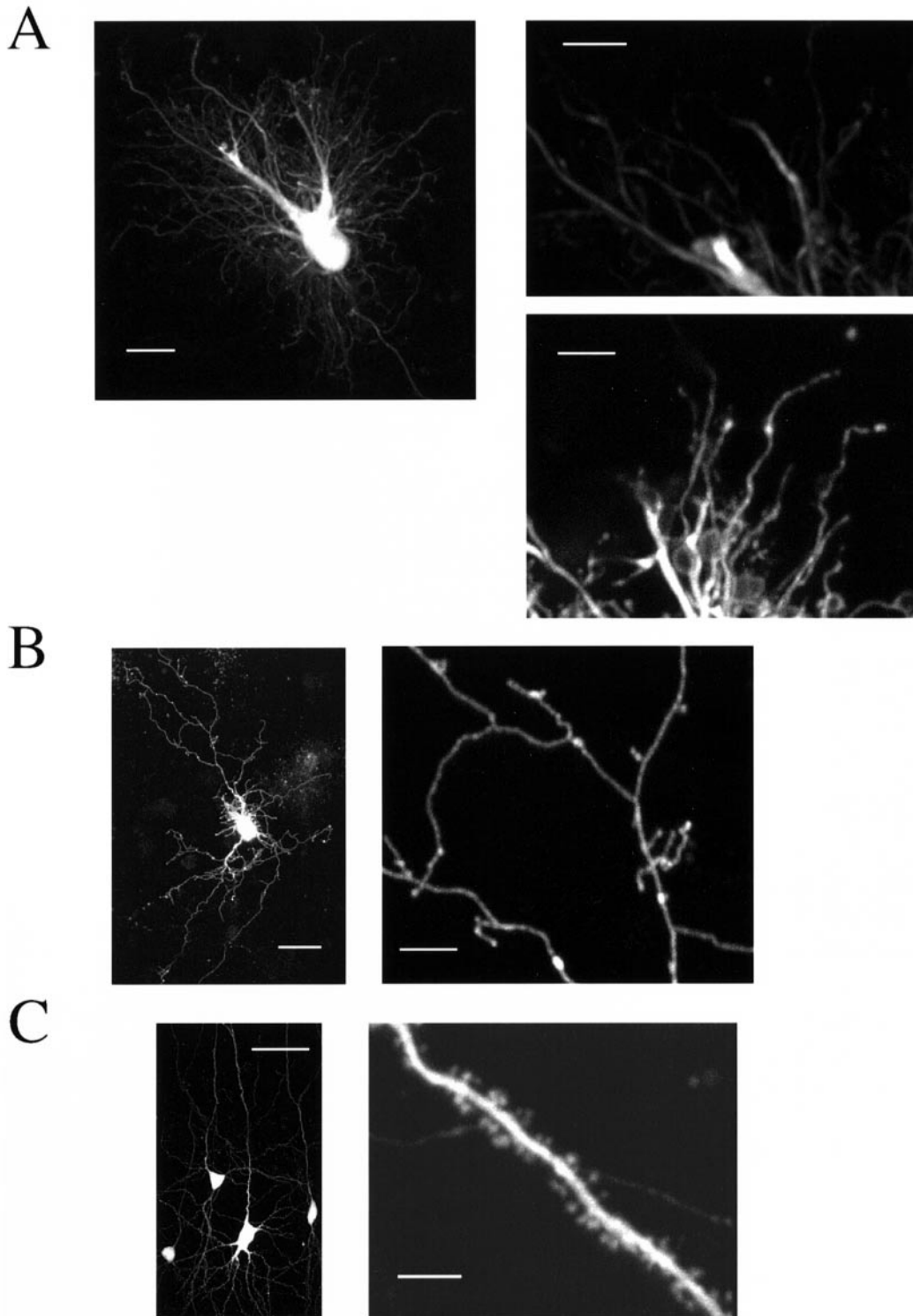


Figure 3. Toxin B-treated neurons. Slice cultures were incubated with 30–150 ng/ml toxin B for 24 h. (A) A subset of neurons had a flurry of thin, long dendrites that extended either from the soma or from the main dendritic trunks. The neuron was a cortical pyramidal cell transfected at 7 DIV and imaged at 10 DIV. (B) Similar phenotype observed in another neuron. Note that the thin dendrites did not taper appreciably and occasionally produced small branches of similar caliber. The neuron was a cortical pyramidal cell transfected at 7 DIV and imaged at 10 DIV. (C) Representative image of a class of neurons which showed an increase in spine density. The neuron was a hippocampal pyramidal cell transfected at 14 DIV and imaged at 16 DIV. Scale bars: 10 μm (A), 25 μm (B) and 50 μm (C) in low magnification, 5 μm in high magnification.

Effect of Toxin B on the Morphology of Spines and Dendrites

To evaluate the general effect of the Rho cascade on the

maintenance of spine morphology, we imaged hippocampal and neocortical pyramidal neurons transfected with GFP after treatment with toxin B, which specifically inactivates all three

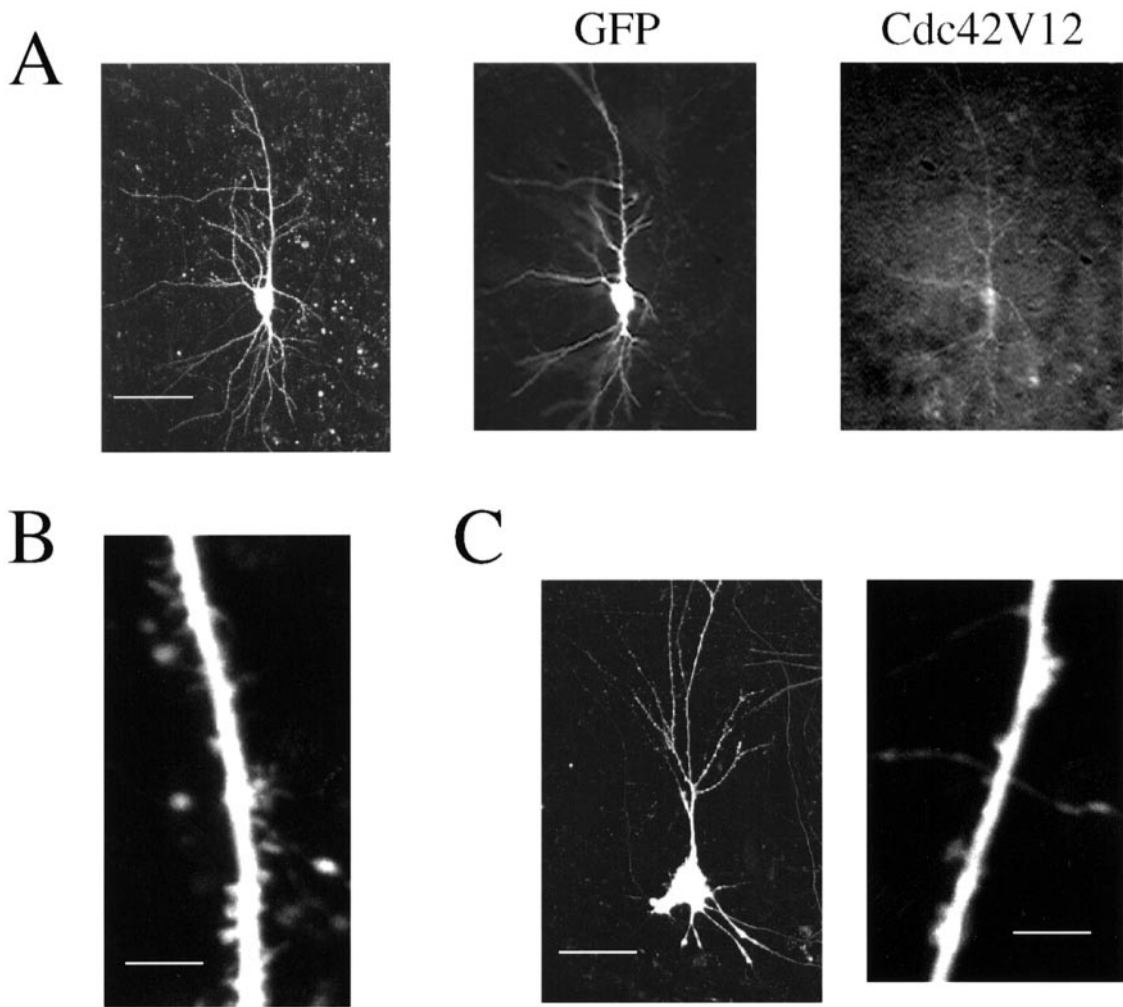


Figure 4. Cdc42V12-transfected neurons. (A) Representative neuron with normal morphology in spine and dendrite. The neuron was a hippocampal pyramidal cell transfected at 10 DIV and imaged at 13 DIV. The images were taken by a two-photon microscope (left) and by a conventional light microscope with an FITC filter (center) or a TRITC filter. (B) High-power images of the neuron in (A). (C) Some neurons have abnormally short dendrites, with swollen tips. Scale bars: 50 μm in low magnification, 5 μm in high magnification.

members of the Rho cascade but not the proteins of the Ras cascade (Just *et al.*, 1995). Bath application of toxin B at concentrations of 30–150 ng/ml for 24 h affected the morphology of dendrites and spines in many cells (Figure 3). The effects on some neurons were very dramatic: instead of normal dendritic morphologies, these cells had an abundance of thin, long dendrites that extended either from the soma or from the main dendritic trunks (12/33 neurons; Figure 3A). The asymmetry in somata and main dendritic trunk indicated that these neurons must have originally belonged to the pyramidal cell class. These small dendrites did not taper appreciably and occasionally produced small branches of similar diameter (Figure 3B). There were either no clearly distinguishable spines in those dendrites or very small structures that could have been very small, stubby spines. Because of this we were unable to quantify the density of spines in these neurons.

Aside from this phenotype, a few cells showed an apparent increase in spine density (4/33 neurons; Figure 3C). Finally, there were many other neurons with apparently normal dendritic or spine morphology (13/33 cells). We concluded from these data that the Rho pathway is involved in the maintenance and rearrangement of dendritic and spine morphology of pyramidal neurons, and decided to explore this pathway in more detail.

Effect of Cdc42 on Spine Morphology

To test whether Cdc42 played a role in the maintenance of spine morphology, we first examined the effect of expressing a constitutively active form of Cdc42 (Cdc42HsV12) in pyramidal neurons (Figure 4). Protein expression was confirmed by immunocytochemistry against myc tags, which showed that Cdc42V12 was distributed throughout the neurons (Figure 4A). Some pyramidal cells (3/5) had normal apical and basal dendrites and spines (Figure 4A,B); however, in other neurons (2/5), some dendrites were abnormally short, as if they were retracting. The tips of these shorter dendrites were swollen (Figure 4C). On affected dendrites spines were stubby (Figure 4C). Nevertheless, pooling together all cells, Cdc42HsV12-expressing neurons had normal values of spine density and length (0.64 ± 0.12 spines/ μm , 1.12 ± 0.10 μm spine length; $n = 163$ spines from five cells).

We examined whether the expression of Cdc42 was necessary for the maintenance of spine morphology, by expressing the dominant negative form of Cdc42 (Cdc42HsN17). No clear effects were found on spine density or length (0.74 ± 0.06 spines/ μm , 1.27 ± 0.08 μm spine length; $n = 362$ spines from 10 cells; Figure 5), although protein expression was confirmed by immunocytochemistry (Figure 5A).

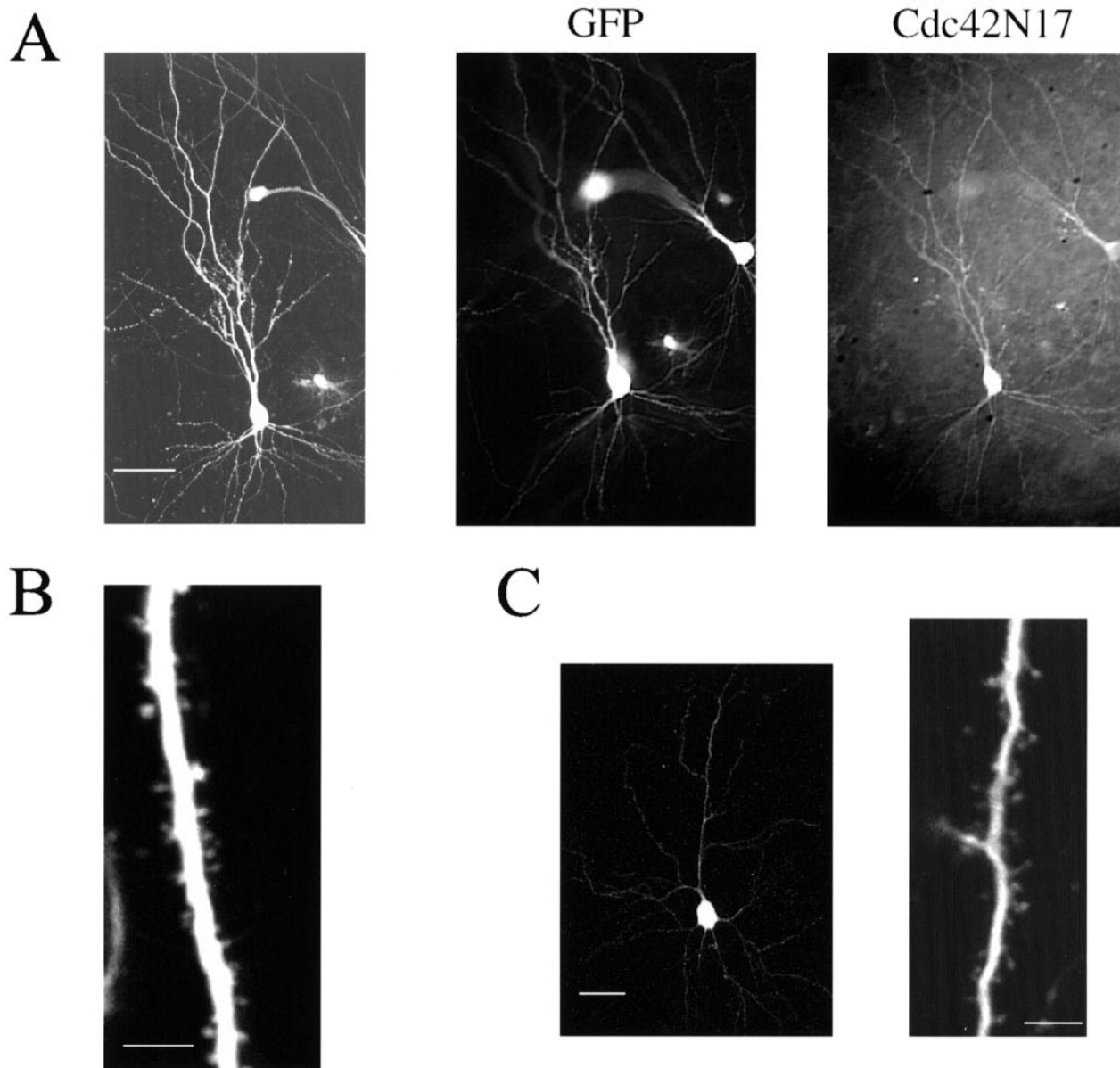


Figure 5. Cdc42N17-transfected neurons. (A) Representative neuron with normal dendritic morphology. The neuron was a hippocampal pyramidal cell transfected at 11 DIV and imaged at 13 DIV. The images were taken by a two-photon microscope (left) and by a conventional light microscope with an FITC filter (center) or a TRITC filter. (B) High-power images of the neuron in (A). (C) Another example of an apparently normal neuron. The neuron was a cortical pyramidal cell transfected at 12 DIV and imaged at 14 DIV. Scale bars: 50 μm in low magnification, 5 μm in high magnification.

We conclude that Cdc42 does not play a major role in the maintenance of spine densities or morphologies. We interpret the abnormal spine morphologies in some Cdc42V12-transfected neurons as a secondary effect produced by the possible retraction of the dendritic trunk.

Effect of Rac on Spine Morphology

We next examined whether Rac played a role in the maintenance of spine morphology. As originally reported by Luo *et al.* (Luo *et al.*, 1996), we found that expression of a constitutively active form of Rac (Rac1V12) had a major effect on spine density and morphology (Figure 6). Every fluorescent neuron had abnormal spines. Most neurons had dendrites covered by lamellipodia-like 'veils' (Figure 6A,B). Some of these veils could be discerned to be

composed of a multitude of very small, thin spines (Figure 6C), so it is possible that all veils were actually not continuous structures but were instead composed of hundreds of small spines. This possibility is supported by data from Rac1V12-transgenic mice, where similar structures were found by electron microscopy to be composed of 'mini' spines (Luo *et al.*, 1996). Because of the small size of these spines, we could not quantify their density, although they appeared to be much higher than normal. These data show that Rac can promote the appearance of spines.

Given the dramatic phenotype observed with RacV12, we wondered if endogenous Rac is involved in spine maintenance. To test this we used a dominant negative form, RacN17. Although many fluorescent cells were found with normal dendritic and spine morphologies (not shown), expression of RacN17 was not

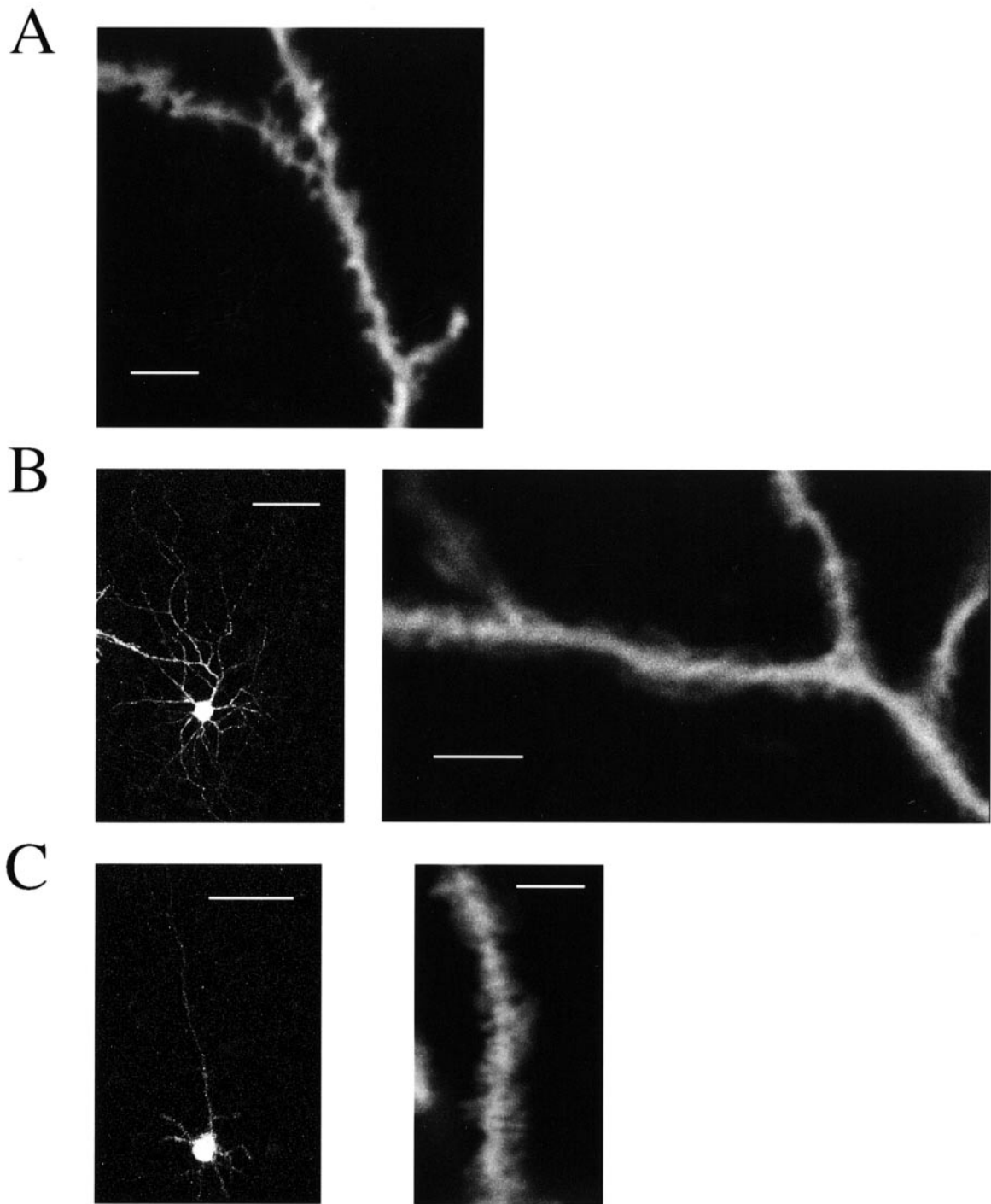


Figure 6. RacV12-transfected neurons. (A,B) Most neurons had dendrites that were covered by lamellipodia-like 'veils'. Neurons were cortical pyramidal cells transfected at 9 DIV and imaged at 11 DIV. (C) Some of these veils could be observed to be composed of a multitude of small spines. This neuron was a cortical pyramidal cell transfected at 9 DIV and imaged at 11 DIV. Scale bars: 50 μm in low magnification, 5 μm in high magnification.

detected by immunocytochemistry, so no conclusions were drawn.

Effect of Rho on Spine Morphology

We next focused our attention on the influence of Rho on spine maintenance. We first tested this by expressing RhoAV14, a constitutively active form. We detected major effects on spine density and morphology (Figure 7). In some neurons, spines

were either present at very low densities or absent (Figure 7A,B; 4/9 cells; 0.15 ± 0.08 spines/ μm , $n = 38$). These cells had clear apical and basal dendrites, which indicated that in spite of being non-spiny neurons they belonged to the pyramidal cell class. The morphologies of the few spines present were also abnormal, the spines having short necks (0.99 ± 0.05 μm , $n = 38$). At the same time, in 5/9 neurons dendrites had spines at normal densities (0.70 ± 0.08 spines/ μm , $n = 349$) and sizes (1.12 ± 0.12 μm , $n =$

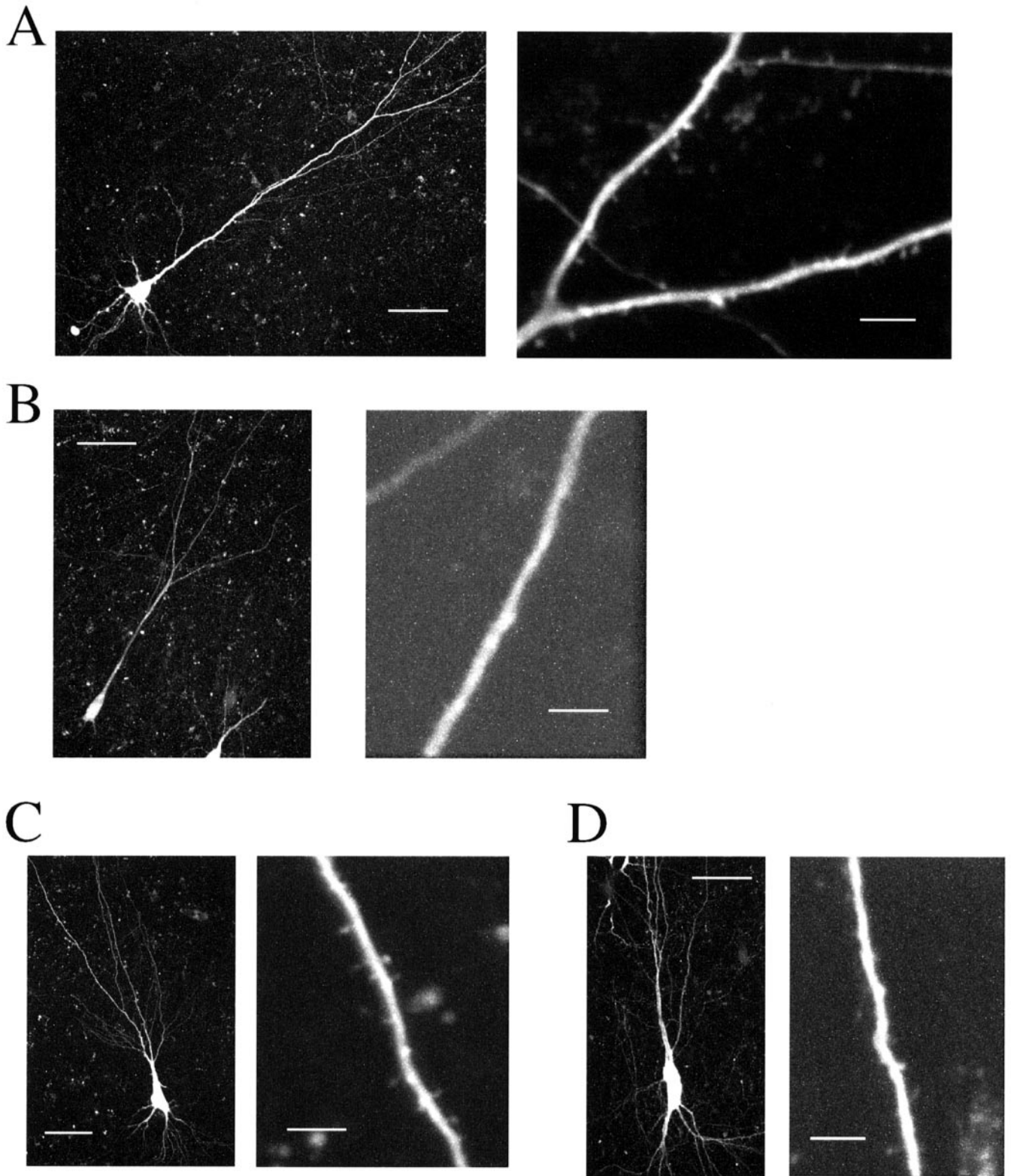


Figure 7. RhoAV14-transfected neurons. In many neurons, spine density was very low (A) or undetectable (B). Most of the spines had short necks (C,D). Neurons were cortical pyramidal cells transfected at 6 DIV and imaged at 8 DIV. Scale bars: 50 μm in low magnification, 5 μm in high magnification.

444). We concluded from these results that Rho expression can repress the maintenance and elongation of spines.

Finally, we examined whether Rho was necessary for spine

maintenance by specifically blocking it by expressing the C3 transferase. C3 transferase is an exoenzyme produced by *Clostridium botulinum* which ADP ribosylates Rho and inhibits

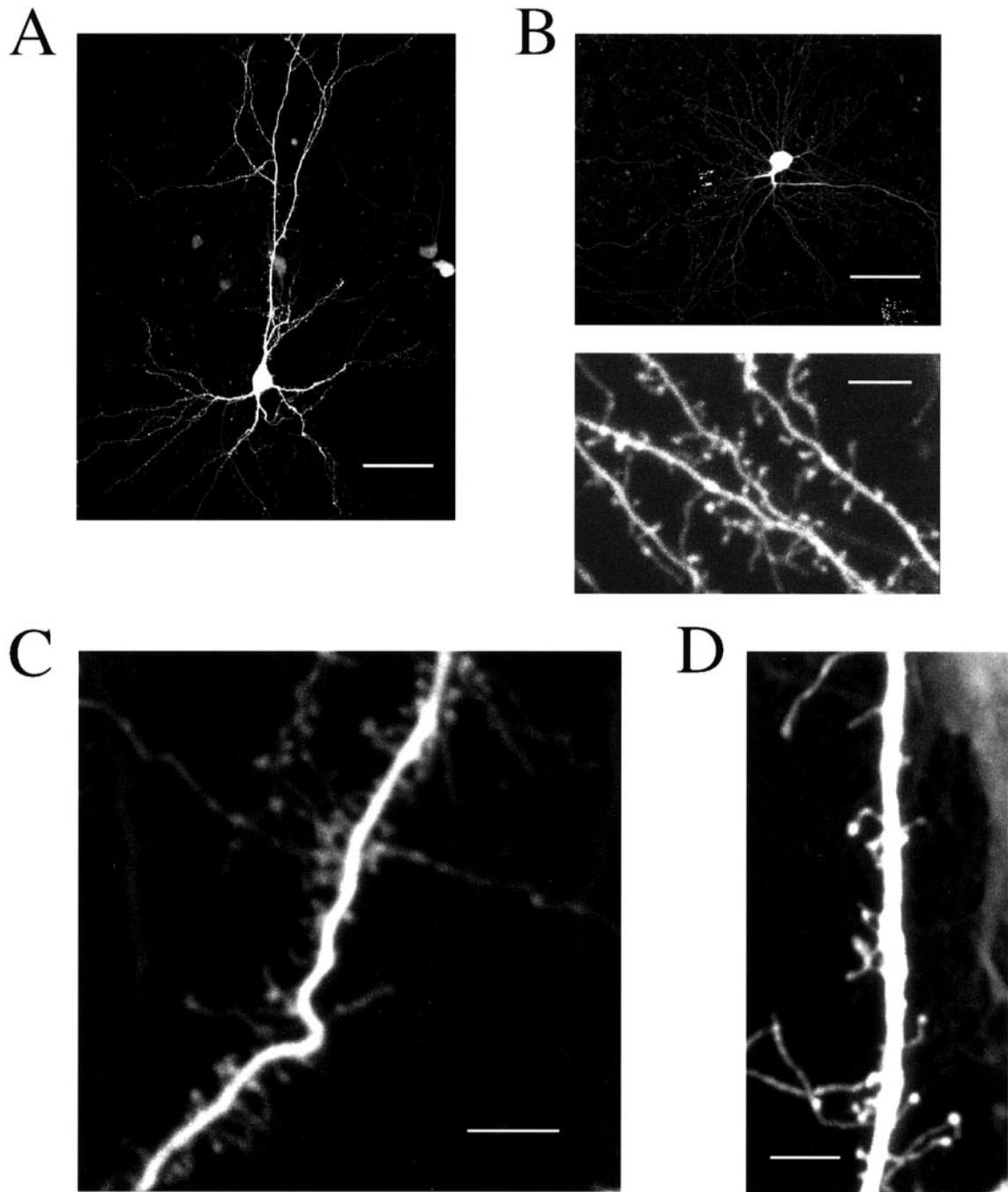


Figure 8. C3 transferase-transfected neurons. (A,B,C) Many neurons showed abnormally high spine density. Neurons were cortical (A,B) or hippocampal (C) pyramidal cells transfected at 18 DIV (A) or 14 DIV (B,C) and imaged at 22 DIV (A) or 16 DIV (B,C). (D) In other neurons, spines had a particularly long neck. This neuron was a cortical pyramidal cell transfected at 18 DIV and imaged at 22 DIV. Scale bars: 50 μm in low magnification, 5 μm in high magnification.

its activity, without affecting Rac or Cdc42 (Aktories *et al.*, 1989; Sekine *et al.*, 1989; Ridley and Hall, 1992; Qiu *et al.*, 1995; Threadgill *et al.*, 1997). In our experience C3 transferase inhibits the activity of Rho more efficiently than a dominant negative RhoN19 construct (A. Minden, unpublished results). While dendritic morphology was normal (Figure 8A), we found two types of abnormal spine morphology; in many neurons there was an abnormally high number of spines, which in some cases completely covered the dendrite (2/7 neurons; 1.1 ± 0.36 spines/ μm ,

$n = 91$; Figure 8B,C). This phenotype resembled some of the neurons transfected with Rac1V12. In other neurons, spines had particularly long necks, resembling filopodia (4/7 neurons; Figure 8D; 2.09 ± 0.1 μm , $n = 138$). These data suggest that Rho has several roles in regulating spine morphology: it prevents spine formation (because its inhibition results in more spines), it promotes spine retraction (because pyramidal cells, which are covered with spines at the ages in which the transfection was done, are found devoid of spines) and it stabilizes shorter spines.

Discussion

Methodological Considerations

In this study we make use of particle-mediated gene transfer (Arnold *et al.*, 1994; Lo *et al.*, 1994), or biolistics, to systematically introduce mutant forms of several proteins in a genetic cascade into neurons in cultured brain slices. As our results and previous work demonstrate (Arnold *et al.*, 1994; Lo *et al.*, 1994), this approach makes genetic studies in brain slices feasible. Part of its usefulness for analysis of the effect of individual genes in neuronal development and function is that this method obviates the need to make transgenic or knockout mice to investigate questions where the phenotype can be appropriately assayed in brain slices. In particular, as we show here, the combination of biolistics with two-photon microscopy (Denk *et al.*, 1994) and GFP expression (Chalfie *et al.*, 1994) enables long-term morphological or functional studies of living neurons after transfection with mutant forms of regulatory proteins. Similar studies could be carried out to characterize genetically or to identify candidate genes involved in the regulation of developmental or physiological processes of neurons.

For this work we concentrated our efforts on examining the role that the Rho family of small GTPases play in the maintenance of spine morphology. Although we have detected some effects on dendritic rearrangements (see Figures 3 and 4C), we imaged neurons at relatively short times after transfection, conditions which may not be optimal to detect in brain slices the pronounced dendritic rearrangements seen in cultured neurons (Threadgill *et al.*, 1997). Nevertheless, these shorter periods in our experiments seem adequate to explore the actions of the Rho proteins in spine morphogenesis. This is based both on the variety of abnormal spine phenotypes encountered and on the very fast morphological rearrangements seen in spines, which can happen in less than a minute (Fischer *et al.*, 1998; Dunaevsky *et al.*, 1999a).

The effects of C3 transferase demonstrate that Rho must be present in the imaged dendrites. Since we cannot confirm the expression of RacN17 in our neurons, we cannot demonstrate with our data that Rac1 is present in dendrites. We have previously encountered difficulties in expressing RacN17 in HeLa and NIH3T3 cells (A. Minden, unpublished experiments). Nevertheless, we think that Rac1 is likely to be present in the dendrites of the imaged neurons because endogenous Rac1 is known to be expressed in hippocampal pyramidal neurons (Olenik *et al.*, 1997) and because RacN17 has effects on the dendrites from cortical pyramidal neurons (Threadgill *et al.*, 1997).

Our study was carried out to assay whether there are major effects of the Rho pathway on spine maintenance. We have not detected any major difference in data from neocortex and hippocampus, so we have consequently pooled our data and conclusions to address the basic regulation of spine morphology in pyramidal neurons. Also, in this study, morphological effects on spines were assayed at the light microscopy level and there are instances, as in the RacV12 data, where the structures were at the limit of our resolution. A combination of GFP transfection with immunoelectron microscopy will allow a proper assessment of the spine morphological phenotypes (Dunaevsky *et al.*, 1999b).

Proposed Model: Antagonistic Effects of Rac and Rho

We have detected a variety of effects of modulating the Rho signaling network on the density and morphology of cortical and hippocampal pyramidal neurons (Table 1; Figure 9). Briefly,

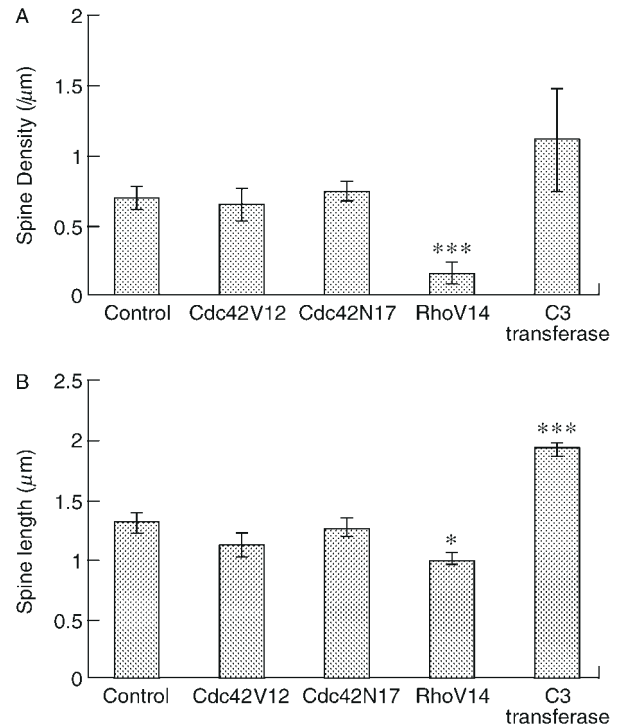


Figure 9. Effect of Rho GTPases on spine density (A) and length (B). For RhoV14 and C3 transferase only data from pyramidal neurons with the clear phenotype were used. Data from RacV12 or toxin B could not be included due to difficulties detecting the spines. Error bars are SEM. *** $P < 0.001$, * $P < 0.05$, t -test.

Table 1

Summary of experiments and detected phenotypes

	Spine density	Spine morphology
Toxin B	fewer, or more	disappeared, or smaller
Cdc42-CA	normal	normal or shorter necks
Cdc42-DN	normal	normal
Rac-CA	increased	'veils', smaller size
Rac-DN	N/A	N/A
Rho-CA	decreased	shorter necks
C3-trans	increased	longer necks

CA = constitutively active; DN = dominant negative. C3-transferase blocks Rho selectively. Toxin B blocks the entire pathway. N/A: not available.

RacV12 (constitutively active) increases spine density while RhoAV14 (constitutively active) reduces it. Also, RacV12 reduces spine size, whereas RhoAV14 reduces spine length and C3 transferase (a Rho blocker) increases spine length. Finally, blocking the entire pathway with toxin B produces different effects on different neurons, with changes both in spine density and morphology. Because of these results, we conclude that the Rho pathway is involved in the maintenance and rearrangement of spine morphology in pyramidal neurons. Our data thus extend to pyramidal cells the published work on effects of RacV12 on Purkinje cells (Luo *et al.*, 1996). Since our experiments were done on developed neurons with extant spines, our conclusions also apply to the maintenance of spine morphology.

We detect two major effects of these pathways: changes in spine density and changes in spine size. These effects appear to be independently modulated by the GTPases. For example, RacV12 increases spine density but decreases spine size, while

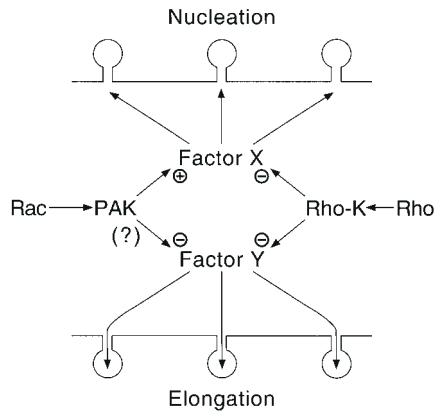


Figure 10. Hypothetical antagonistic model of action of Rac and Rho on spines.

RhoAV14 decreases both density and size. This suggests that the density and size of spines are controlled independently by two different mechanisms. Based on our results, we propose a model in which Rac and Rho have antagonistic effects: Rac promotes the development of new spines while Rho appears to block both their formation and maintenance (Figure 10). This antagonistic model could explain why toxin B can have a milder phenotype than some of the Rac or Rho transfections, because blocking the entire cascade might be less damaging than blocking part of it. The antagonistic effect could also explain why some of the toxin B neurons had more spines: perhaps in those cells the toxin inhibited Rho more reliably. Nevertheless, we cannot rule out that the toxin B phenotype is due to the relatively low doses of toxin used or because of differences between the two different experimental manipulations (bath application versus cell-specific transfection).

Our proposed model is consistent with previous reports that have shown that Rac and Cdc42 can antagonize Rho during cell movement, where Rac and Cdc42 promote protrusions at the leading edge of cells while Rho induces retraction of the leading edge. Likewise, in some cells Rho induces an increase in stress fiber formation while Rac and Cdc42 lead to a decrease in stress fibers (Dutartre *et al.*, 1996; Manser *et al.*, 1997; Burridge, 1999). The opposing roles of Rac and Cdc42 are probably due to at least in part to their opposite roles in regulating myosin light chain phosphorylation. Rho is thought to act, via the Rho kinase, to phosphorylate and thereby inhibit myosin phosphatase. This results in increased myosin light chain phosphorylation, which is associated with an increase in stress fiber formation and contractility. In contrast, Rac and Cdc42 act via the serine/threonine kinase PAK, to phosphorylate and thereby inactivate myosin light chain kinase. This leads to a decrease in myosin light chain phosphorylation, and a decrease in stress fibers and contractility (Sanders *et al.*, 1999; van Leeuwen *et al.*, 1999).

We hypothesize that a similar antagonism occurs in dendritic spines. The different effects that Rac and Rho have on both spine density and spine size could be due to two molecular mechanisms (Figure 10). We propose that Rac and Rho have opposite effects on a common target (factor X, or nucleating factor) that is present at the dendrite and is involved in nucleating the position of the future spine. Our data suggest that Rac may positively regulate factor X, while Rho has a negative regulatory role. Since Rac and Rho generally do not share the same direct targets, factor X would most likely be an indirect target. This would be analogous to myosin light chain, which is regulated indirectly by

Rac and Rho via PAK and Rho kinase, respectively. A second factor (factor Y; elongation factor) would be active at the spine itself and would be responsible for its length and size (Figure 10). This factor may be inhibited only by Rho, because the reduction of spine size observed with RacV12 might be a consequence of the high number of spines. Factor Y could potentially be a direct Rho target, such as Rho kinase, or may be an indirect target of the Rho pathway. Future experiments will explore the identity of these putative target molecules and the mechanisms by which they regulate spine density and morphology.

Notes

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