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Differential effects of Rho GTPases on axonal and dendritic development in hippocampal neurones

G. Ahnert-Hilger,* M. Höltje,* G. Große,* G. Pickert,* C. Mucke,* B. Nixdorf-Bergweiler,† P. Boquet,‡ F. Hofmann§ and I. Just§

*Centrum für Anatomie, Charité Universitätsmedizin, Berlin, AG Funktionelle Zellbiologie, Berlin, Germany

†Zoologisches Institut der Christian Albrechts-Universität Kiel, Kiel, Germany

‡Inserm U. 452, Faculté de Médecine, Nice, France

§Institut für Toxikologie der Medizinischen Hochschule Hannover, Hannover, Germany

Abstract

Formation of neurites and their differentiation into axons and dendrites requires precisely controlled changes in the cytoskeleton. While small GTPases of the Rho family appear to be involved in this regulation, it is still unclear how Rho function affects axonal and dendritic growth during development. Using hippocampal neurones at defined states of differentiation, we have dissected the function of RhoA in axonal and dendritic growth. Expression of a dominant negative RhoA variant inhibited axonal growth, whereas dendritic growth was promoted. The opposite phenotype was observed when a constitutively active RhoA variant was expressed. Inactivation of Rho by C3-catalysed ADP-ribosylation using C3 isoforms (*Clostridium limosum,* $C3^{lim}$ or *Staphylococcus aureus*, $C3^{stau2}$), diminished axonal branching. By contrast, extracellularly applied nanomolar concentrations of C3 from *C. botulinum* ($C3^{bot}$) or enzymatically dead $C3^{bot}$ significantly increased axon growth and axon branching. Taken together, axonal development requires activation of RhoA, whereas dendritic development benefits from its inactivation. However, extracellular application of enzymatically active or dead $C3^{bot}$ exclusively promotes axonal growth and branching suggesting a novel neurotrophic function of C3 that is independent from its enzymatic activity.

Keywords: axonal development, dendritic development, enzymatically dead C3, neurotrophic effects, Rho proteins.
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During development, neurones undergo dramatic morphological changes that culminate in the differentiation of axonal and dendritic arbours. One of the first discernible steps is the generation of thin processes or neurites that elongate by the activity of growth cones at their tips. This early developmental state is also referred to as stage 2 and lasts about 24 h. Within a few hours later, during stage 3, one of these neurites begins to grow rapidly and differentiate into an axon. During stage 4, after 3–4 days, the other neurites develop into dendrites (Goslin and Banker 1989). Once this decision is made, axons and dendrites grow and arbourize more or less extensively, depending on the type of the neurone. These morphological changes require a permanent reorganization of the neuronal actin and microtubule network.

In recent years it has become clear that Rho proteins play an essential role in the differentiation of neuronal processes (Bradke and Dotti 1999). Rho proteins are represented by a subfamily of small GTPases, including Rho, Rac and Cdc42, that are considered as master regulators of the cytoskeleton (Threadgill *et al*. 1997; Hall 1998; Yamashita *et al*. 1999; Luo 2000). However, it is not clear which precise role Rho GTPases have during the developmentally advanced steps of elongation and arbourization of axons and dendrites. In particular, it is not understood how activation or inactivation of Rho proteins influences growth and arbourization of axons in comparison to that of dendrites.

Neurotrophins that regulate neuronal morphogenesis have been shown to play comparable roles in axon and dendritic

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Address correspondence and reprint requests to Dr Gudrun Ahnert-Hilger, Centrum für Anatomie, Charité Universitätsmedizin, Berlin, AG Funktionelle Zellbiologie Berlin, Philippstr. 12, D-10115 Berlin, Germany.
E-mail: gudrun.ahnert@charite.de

Abbreviations used: DIV, days *in vitro*; EGFP, enhanced green fluorescence protein; MAP2, microtubule associated protein 2; NFP, neurofilament protein; PBS, phosphate-buffered saline.

development, suggesting that axonal and dendritic growth cones may share similar signal transduction machineries while others like semaphorin A affect axons and dendrites differently (for review see Scott and Luo 2001). Most of these receptor-mediated effects are transduced by members of the Rho family, including Rho, Cdc42 and Rac (for review see Scott and Luo 2001; Luo 2002). However, reports, especially on RhoA effects, are quite contradictionary. Growth of undifferentiated neurites (stage 2) and of those that have started to differentiate to an axon (stage 3) was promoted by either activating or inhibiting members of the Rho GTPase family (Hall 1998; Yamashita *et al*. 1999; Luo 2000). Lysophosphatidic acid (LPA), a constituent present in serum, promotes neurite retraction through a $G\alpha_{12/13}$-mediated activation of Rho (Kranenburg *et al*. 1999). Activation of RhoA has been shown to promote dendritic and axonal growth in rat cortical neurones cultivated in the presence of serum (Threadgill *et al*. 1997). Thus, RhoA appears to have variable effects on all types of processes depending on the developmental stage of the neurone and the culture condition, especially the presence or absence of serum. Most of the studies have focussed on growth cone development and neurite formation or have measured axon elongation after very short periods of cultivation (after 4–24 h) where axons cannot be differentiated from dendrites with certainty or promoting effects may have escaped detection. Studies concerning the differential effects that Rho GTPases might have on either axon or dendrite elongation or branching, which occur later during development, are missing so far. Serum, which contains a variety of factors including LPA and which is often used for the cultivation of primary neurones, may also obscure the picture.

In addition, not only the developmental time window but also the mixture and local concentrations of Rho GTPases appear to be critical. In this respect, it has been shown that both Cdc42 and RhoA may mediate attraction and repulsion of growth cones dependent on the activity of protein kinase A and myosin, suggesting a complex cross-talk between these two proteins (Yuan *et al*. 2003). In cerebellar granule cells, different amounts of RhoA appear to inhibit or enhance neurite/axon growth either by activating ROCK or by activating mDia (mammalian homologue of the *Drosophila* gene Diaphanous), respectively (Arakawa *et al*. 2003). Although contradictionary, all the data underscore the central role Rho GTPases, especially Rho A, play during the differentiation of neurones.

The switch from an undifferentiated neurite to a dendrite or an axon and their further differentiation is a feature that can be studied in primary culture of isolated neurones and that takes days more than hours. The time course of an axonal/dendritic differentiation critically depends on the cultivation conditions and the brain area the neurones are derived from. Using serum-free primary cultures of mouse hippocampal neurones cultivated long enough to allow for differentiation of axons and dendrites, we investigated how RhoA activity regulates the developmentally later steps of axonal and dendritic growth and branching.

Experimental procedures

Recombinant C3 proteins, C3-EGFP cDNA vectors and RhoA EGFP-vectors

Recombinant proteins

The gene of *Clostridium botulinum* C3 (accession No. X59039) or mutants of C3 harbouring glutamine (Q) or alanine (A) instead of glutamate (E) at position 174 ($C3^{bot}E174A$, $C3^{bot}E174Q$) and the gene of *C. limosum* transferase (accession No. X872155), were cloned into pGEX expression vector. After expression in *Eschrichia coli,* GST-C3 fusion proteins were affinity-purified on glutathion-Sepharose followed by thrombin cleavage to obtain C3 proteins. The gene of *Staphylococcis aureus* transferase strain HMI (accession No AJ277173) was cloned into pQE30. After expression in *E. coli,* His-tagged transferase were purified on nickel-columns. As all C3 proteins either with or without enzymatic activity were purified from an identical *E. coli* expression system a contamination of one single recombinant protein can be definitively excluded.

For transfection, the gene of *C. botulinum* C3 (accession No. X590399), the mutant forms $C3^{bot}E174A$ and $C3^{bot}E174Q$ were cloned into pEGFP eukaryotic expression vector. The genes for wild-type, dominant negative (RhoA N19), or constitutive active (RhoA V14) RhoA were cloned into pEGFP eukaryotic expression vector.

Cell culture

Hippocampal neurones were prepared from 17-day-old fetal NMRI mice. In some experiments, day 16 fetal mice were also used. Dissected pieces of hippocampi were rinsed twice with phosphate-buffered saline (PBS), then with dissociation medium [modified essential medium (MEM) supplemented with 10% fetal calf serum, 100 IE insuline/L, 0.5 mM glutamine, 100 U/mL penicillin/streptomycin, 44 mM glucose and 10 mM HEPES buffer] and dissociated mechanically. The suspension was centrifuged at 210 *g* for 2 min at 21°C, redissociated in starter medium (serum-free neurobasal medium supplemented with B27, 0.5 mM glutamine, 100 U/mL penicillin/streptomycin and 25 μM glutamate) and plated on coverslips pre-coated with 0.5% poly-L-lysine followed by 40 μg/mL collagen dissolved in PBS layered in 24-multiwells at a density of 20 000 cells/well. All ingredients were obtained from Gibco/BRL Life Technologies (Eggenstein, Germany). Neurones were cultivated up to 14 days *in vitro* (DIV) in an humidified atmosphere with 10% CO_2. One day after plating neurones (DIV1), the various C3 proteins were added to the culture medium at the indicated concentrations. Five days later (DIV6), neurones were fixed with 4% formaldehyde dissolved in 0.1 M phosphate buffer pH 7.4. Fixed cells were treated with PBS for 15 min and subsequently permeabilized for 30 min at room temperature (RT) using 0.3% Triton X-100 dissolved in PBS. Neurones were stained by a monoclonal antibody against neurofilament protein (NFP, 200 kDa, final dilution 1 : 200) or polyclonal antibodies against microtubule-associated proteins 2 (MAP2, final dilution 1 : 500; both from

Chemicon International, Hofheim, Germany) to mark axons or dendrites, respectively. Immunoreactivity was visualized using anti-mouse IgG coupled to Cy2 (Jackson Immuno Research Laboratories, West Grove, PA, USA) and neurones were morphometrically analysed as given below.

Hippocampal primary cultures were transfected using the cDNA vectors indicated. Transfection of neurones was performed at DIV4 with 0.3 μg cDNA/15 mm dish using the Effectene system (Life Technology) according to the manufacturer's description. Transfected neurones were fixed after 24 h at DIV5 and transfected cells visualized by the green fluorescence protein were morphometrically analysed as given below.

Morphometrical analysis

Photomicrographs were taken from individual neurones and total length as an indicator of growth as well as number of branches from axons as well as dendrites as indicated were analysed morphometrically using the Neurolucida software (MicroBrightField., Inc). Axon length encompasses all visible parts of an axon including the length of its branches. Dendrite length indicates the length of all dendrites including their branches, while dendrite branching indicates primary as well as secondary dendrites.

Each experiment involving neurones from one preparation of embryonic hippocampus was designed with controls and the various treatments. To compare the effects of all C3 isoforms data from 11 experiments were combined and statistically analysed using Student's *t*-test. The data were then subjected to an α adjustment according to Bonferoni–Holm with α, the interval of confidence, limited to 5%, a method used when pooled controls from different experiments were compared to treatments not performed in all the individual experiments.

ADP ribosylation reaction

Hippocampal primary cultures were treated with the various C3 proteins for 5 days. Then the medium was removed and cells were frozen at −80°C. The frozen cultures were scraped off and homogenized followed by centrifugation (10 min at 2000 *g*) to prepare the post-nuclear supernatant, that was subjected to *in vitro* ADP-ribosylation using $C3^{bot}$ protein. To this end, 25 μg of hippocampal lysate proteins were incubated in the presence of 50 mM HEPES buffer pH 7.4 supplemented with 2 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM NaCl and 0.3 μM [P-32]NAD at 37°C for 15 min. Thereafter, Laemmli sample buffer was added, boiled for 10 min at 95°C and subjected to 12.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). After staining, destaining and drying of the gel, radioactivity was analysed by phosphorimager (Cyclone, Packard Instruments, Meriden, CT, USA).

Results

Axonal and dendritic growth and branching are influenced by constituents of the medium, co-cultivated non-neuronal cells and extracellular matrices. To reduce variability in cell culture conditions, we cultivated hippocampal neurones on an inert poly-L-lysine/collagen coat, in the absence of serum to minimize glial contaminations as well as influence of serum constituents. In mouse hippocampal neurones cultivated under these conditions for 14 days (DIV14), axons can be clearly distinguished from dendrites by their morphology and by differential distribution of NFP and MAP2. Differential distribution, however, was not detectable during the early phases of culture (i.e. after 1 or 2 DIV) where both putative axonal and dendritic markers are distributed all over the cell. Differential labeling of processes was first visible at DIV3 and was clearly discernible in all neurones after DIV6 (Fig. 1).

In order to find out whether and how the RhoA GTPase affects the developmentally advanced steps of axonal and dendritic elongation we performed two series of transfection using cultures obtained from E17 or E16 mouse embryos with RhoA constructs fused to the enhanced green fluorescence protein (EGFP) coding either for wild-type (EGFP-RhoA), dominant negative (EGFP-RhoA N19), or constitutive active (EGFP-RhoA V14) Rho proteins. Transfection was performed on DIV4 and axons or dendrites were analysed at DIV5. It turned out that the expression of EGFP-RhoA N19 dramatically reduced axon branching and had also negative effects on axon length. In contrast, expression of EGFP-RhoA (wild-type) or EGFP-RhoA V14 has no effect (Figs 2a and b). The contradiction between the strong effect of dominant negative but the poor effect of constitutive

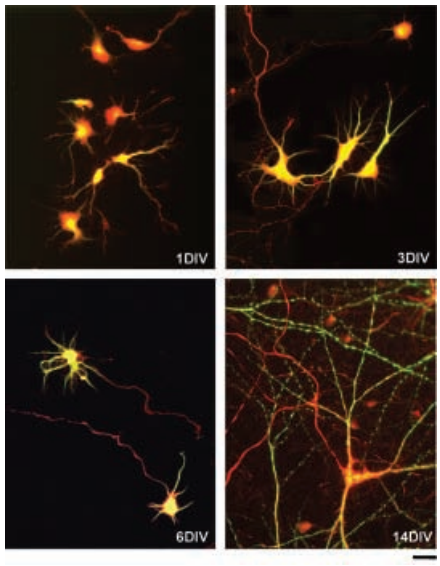

Fig. 1 Development of axonal and dendritic differentiation in mouse hippocampal primary culture. Mouse hippocampal neurones from embryonic day 17 were seeded at a density of 2×10^{-4}/well (113 mm^2) and cultivated in serum free medium. After one, three, six or 14 days in culture (DIV 1, DIV 3, DIV 6, DIV 14) neurones were fixed and double-immunostained with a monoclonal antibody against MAP2 (green) and a polyclonal antibody against NFP 200 (red). Note that at DIV 3 and DIV 6 the NFP-positive but MAP2-negative axon is clearly discernible. Scale bar: 20 μm.

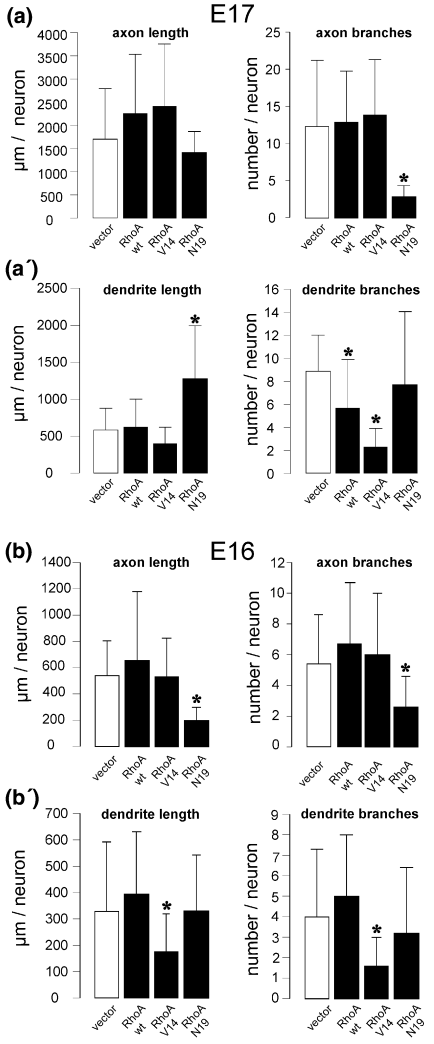

Fig. 2 Differential effects of EGFP-RhoA constructs on axonal and dendritic growth. Wild-type RhoA, the dominant negative RhoA N19 and the constitutive active RhoA V14 proteins were expressed as fusion proteins with the enhanced green fluorescent protein (EGFP). Hippocampal neurones [obtained from mice embryos at day 17 (E17) or day 16 (E16) and seeded at a density of 2×10^{-5}/well] were transfected with either mere EGFP-vector, EGFP-RhoA, EGFP-RhoA N19 or EGFP-RhoA V14 at DIV4 using the Effectene transfection kit. Neurones were fixed at DIV5 and analysed morphometrically by the Neurolucida software. (a, b) Morphometric analysis of for axonal length and branching. (a′, b′) Morphometric analysis of neurones for dendritic length and branching. Values include measurements from 12 (E17) or 19 (E16) neurones for vector, 17 (E17) or 14 (E16) for wild-type RhoA, 17 (E17) or 17 (E16) for RhoA V14 and 9 (E17) or 18 (E16) for RhoA N19. Statistical significance ($p < 0.05$) is indicated by an asterisk.

active RhoA (Figs 2a and b) may be based on the saturation of the signal transduction pathway positively controlling axonal development with intrinsic RhoA; thus, the increase of cellular RhoA concentration by transfection has little effects, whereas its functional inhibition by RhoA N19 results in a potent reversal.

A totally different picture was obtained when we analysed the effects of these mutant Rho forms on dendrites. In contrast to its inhibitory effects on axon growth, expression of RhoA N19 significantly increased dendritic length in E17-derived neurones without having considerable effects in the second experiment from E16 neurones, whereas RhoV14 that has only minor effect in the first but significantly inihibited dendritic growth and branching in the second experiment (Figs 2a′ and b). These variations may reflect changes in the local concentrations in individual neurones. Probably RhoA-sensitive pathways develop before the upregulation of RhoA. While in the experiment from E17, neurones already have high intrinsic RhoA concentrations, in the experiment from E16, neurone upregulation has not yet started, resulting in low local concentration of RhoA. Under the latter condition only RhoV14 was able to cause negative effects on dendritic growth while – after the developmental local upregulation of RhoA has started – additional active RhoA has less effects on dendritic growth. However, RhoN19 could functionally antagonize the intrinsic RhoA. Probably the local RhoA upregulation is sensitively controlled at this developmental stage. Variations in the development between the different neuronal subpopulation obtained in hippocampal cultures may also be involved. Irrespective of these variations, the data clearly indicate that RhoA promotes axonal and inhibits dendrite development.

Next we analysed whether a selective inactivation of RhoA by C3-catalysed ADP-ribosylation exerts comparable effects on axonal and dendritic growth. C3 proteins, specifically, ADP-ribosylate RhoA, -B and -C GTPases (Boquet *et al*. 1998; Just *et al*. 2001) and are produced by *C. botulinum* C3 ($C3^{bot}$; Rubin *et al*. 1988; Aktories *et al*. 1989; Moriishi *et al*. 1993), by *C. limosum* ($C3^{lim}$; Just *et al*. 1992), as well as by *S. aureus* ($C3^{stau}$; Sugai *et al*. 1992; Wilde *et al*. 2001). All C3 proteins are isoforms possessing a certain degree of homology at amino acid level, but they all share the catalytic amino acid glutamate (Glu174 in $C3^{bot}$); exchange of this glutamate results in enzymatically dead C3 proteins (Aktories *et al*. 2000). In order to ensure a differentiation between axons and dendrites, neurones were treated with C3 proteins at DIV1 and analysed at DIV6.

Purified ($C3^{bot}$ pur.) or recombinant C3 ($C3^{bot}$) promoted axon growth and branching when applied to hippocampal neurones at a concentration of 20 nM. These effects, however, were not shared by the related recombinant transferases $C3^{lim}$ and $C3^{stau2}$ that reduced axon branching under similar experimental conditions (Figs 3a and b). In one individual experiment, included in the combined data in Fig. 3(b), treatment with 20 nM $C3^{lim}$ significantly reduced axon growth from 790.1 ± 244.4 µm (controls $n = 11$) to 483.4 ± 250.1 µm ($C3^{lim}$ $n = 11$, $p = 0.004$) and branching from 4.6 ± 1.7 to 2.5 ± 1.8 ($p = 0.006$). Unexpectedly, the

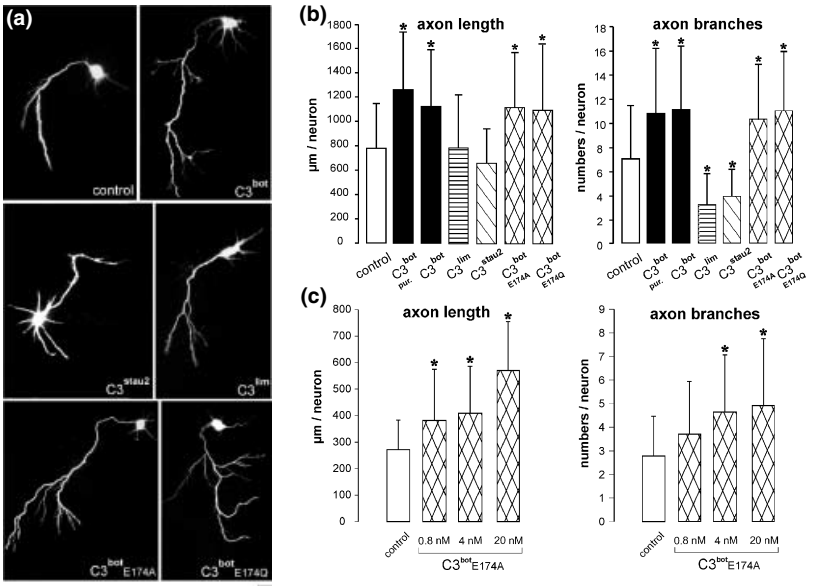

Fig. 3 Effects of C3-isoforms on axonal growth of hippocampal neurones. (a) Primary cultures of mouse hippocampal neurones were prepared at embryonic day 17 and seeded at a density of 2×10^{-4}/well (113 mm^2). The indicated C3 isoforms were applied at DIV1 at a concentration of 20 nM for 5 days. Neurones were stained at DIV6 with antineurofilament (NFP) protein (200 kDa) antibody and photomicropgraphs from individual neurones were subjected to morphometrical analysis using Neurolucida software. Scale bar: 20 µm. $C3^{bot}$ pur., C3 purified from *C. botulinum;* $C3^{bot}$, recombinant $C3^{bot}$; $C3^{lim}$, recombinant C3 from *C. limosum;* $C3^{stau2}$, recombinant C3 from *S. aureus* strain HMI; $C3^{bot}$ E174A and $C3^{bot}$ E174Q, enzymatically dead C3bot. (b) The bars give the combined morphometric analyses (mean values ± SD) obtained from a variety of neuron preparations (see below) for the effects of 20 nM of each C3 protein on axon length (left panel) and axon branches (right panel). The following number of neurones were analysed for each condition: control: 256 neurones from 11 preparations; $C3^{bot}$ pur. 181 neurones from eight preparations; $C3^{bot}$: 142 neurones from four preparations; $C3^{lim}$: 59 neurones from five preparations; $C3^{stau2}$: 29 neurones from two preparations; $C3^{bot}$ E174A: 147 neurones from four preparations; $C3^{bot}$ E174Q: 75 neurones from three preparations. The respective *p*-values (Student's *t*-test) calculated against control are for axonal length: $C3^{bot}$ pur.: 1.81E-25; $C3^{bot}$: 1.3E-12; $C3^{bot}$ E174A: 7.2E-13; $C3^{bot}$ E174Q: 7.2E-6: In contrast, the related recombinant proteins $C3^{lim}$ and $C3^{stau2}$ had no effect or rather slightly decreased axonal growth (*p*-values 0.9 or 0.04, respectively). A similar picture emerged when analysing axonal branching. Again only $C3^{bot}$ and the two enzymatically dead $C3^{bot}$ proteins significantly increased the number of branches with the following *p*-values calculated against controls: $C3^{bot}$ pur. 3.14E-13; $C3^{bot}$: 3E-13; $C3^{bot}$ E174A: 5.6E-11; $C3^{bot}$ E174Q: 8.2E-9. Similar to axonal length, axonal branching was significantly decreased by the recombinant $C3^{lim}$ ($p = 2.4E-14$) and $C3^{stau2}$ ($p = 5.8E-8$). From the various C3 proteins with ADP-ribosylating activity ($C3^{bot}$ pur.) and the recombinant proteins ($C3^{bot}$, $C3^{lim}$ and $C3^{stau2}$) tested, exclusively $C3^{bot}$ showed the neurotrophic property. This neurotrophic property was still present in the enzymatically dead (i.e. non-Rho ADP-ribosylating) $C3^{bot}$ E174A and $C3^{bot}$ E174Q proteins Thus, $C3^{bot}$ and its enzymatically dead forms are capable of promoting axonal growth and branching.α-values were adjusted according to Bonferoni–Holm (six conditions and α limited to 0.05) and significance was marked by an asteriks. (c) The axonal growth promoting effects of $C3^{bot}$ E174A are discernible at concentration of about 1 nM. The following *p*-values calculated against control (24 neurones) were for axon length: 0.019 (0.8 nM, 25 neurones); 0.011 (4 nM, 23 neurones); 1.9E-7 (20 nM, 23 neurones) and for axon branches: 0.122, 0.006 and 0.005, respectively. α-values were adjusted according to Bonferoni–Holm (three conditions and α limited to 0.05) and significance was marked by an asteriks.

mutant $C3^{bot}$ devoid of any ADP-ribosyltransferase activity – generated by an exchange of the catalytic amino acid glutamate to either alanine ($C3^{bot}$ E174A) or glutamine ($C3^{bot}$ E174Q) – had the same promoting effect on axon growth and branching (Figs 3a and b). In another individual experiment included in the data presented in Fig. 3(b), treatment with 20 nM of recombinant $C3^{bot}$, $C3^{bot}$ E174Q, or $C3^{bot}$ E174A significantly increased ($p < 0.01$) axon growth from 687.4 ± 251.7 (control $n = 82$) to either 1024.6 ± 281 ($n = 69$), or to 1108.4 ± 292.1 ($n = 56$), or to 1065.1 ± 295 ($n = 63$) and axon branching ($p < 0.002$) from 7.4 ± 3.8 (control) to either 9 ± 3.8, or to 10.1 ± 4.2, or to 9.3 ± 3.9, respectively. The axonotrophic effect of enzymatically dead $C3^{bot}$ was discernible at a concentration of 1 nM (Fig. 3c). After 5 days of treatment with $C3^{bot}$ E174A (at DIV6), only the NFP-immunopositive but MAP2-immunonegative axons were longer compared to untreated controls. The enzymatically dead $C3^{bot}$ proteins in fact did not ADP-ribosylate intraneuronal Rho, not even after 5 days of incubation. By contrast, all enzymatically active C3 proteins applied at 20 nM exhibited ADP-ribosylation of neuronal Rho, most pronounced by $C3^{bot}$, as shown by differential

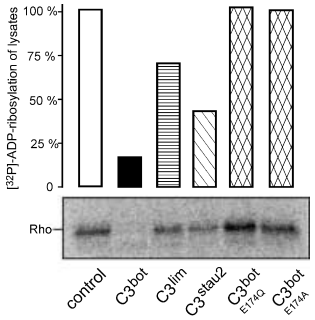

Fig. 4 Differential ADP-ribosylation of hippocampal cultures pretreated with C3-isoforms. Hippocampal primary cultures were incubated at DIV1 without any addition (control) or with recombinant proteins $C3^{bot}$, $C3^{lim}$, $C3^{stau2}$, $C3^{bot}$ E174QA, and $C3^{bot}$ E174Q (each 20 nM corresponding to 480 ng/mL) for 5 days. Thereafter, cultured cells were washed, homogenized and 10 μg of the post-nuclear supernatant was ADP-ribosylated by $C3^{bot}$ in the presence of [P-32]NAD. The samples were separated on 12.5% SDS–PAGE and analysed by phosphorimager (lower panel). The rational of this assay is that a decrease in incorporation of radioactivity in the Rho band reflects the *in vivo* ADP-ribosylation in the intact cell. The diagram (upper panel) gives the evaluation of phosphorimager data. Enzymatically active C3-proteins ($C3^{bot}$, $C3^{lim}$, $C3^{stau2}$) are capable of ADP-ribosylating intracellular Rho proteins, whereby $C3^{bot}$ is the most efficacious one. The enzymatically dead $C3^{bot}$ E174Q and $C3^{bot}$ E174A mutants did not result in a decreased differential ADP-ribosylation indicating loss of enzymatic activity also under *in vivo* conditions.

ADP-ribosylation (Fig. 4). Thus, the neurotrophic effects of enzymatically dead $C3^{bot}$ E174A and $C3^{bot}$ E174Q are definitely not mediated by a direct inhibition of Rho GTPases.

Hippocampal neurones prepared from E17 may be contaminated by astrocytes that can reach up to 10% of the total number of cells, as counted from staining with glial fibrillary protein. Astrocytes release trophic factors like glial-derived growth factor or S100β, which may mediate the axonotrophic effects observed after application of enzymatically dead $C3^{bot}$ E174A and $C3^{bot}$ E174Q. However, when using cortical neurones prepared from E14 days that contained almost no astrocytes (below 1%) $C3^{bot}$ E174Q exhibited similar effects on axon growth and branching (not shown). In addition, S100β a general astrocyte marker that is exclusively released by astrocytes and has been reported to increase neurite growth of serotonergic neurones (Azmitia *et al.* 1990) did not affect axon growth and branching of hippocampal neurones (Hopf *et al.*, manuscript yet to be published). The specificity of extracellularly applied $C3^{bot}$, $C3^{bot}$ E174A and $C3^{bot}$ E174Q is further underlined by the fact that other isoforms of C3 with functional ADP-ribosyltransferase activity but differing amino acid sequence exerted opposing effects on axonal growth and branching (Figs 3a and b). While the data obtained with $C3^{lim}$ and $C3^{stau2}$ correlate with the negative effects on axon growth observed after transfection with Rho N19, $C3^{bot}$ and its enzymatically dead forms appear to have additional axon promoting effects.

To distinguish more precisely between extracellular (non-Rho-mediated) and intracellular effects (involving ADP-ribosylation), hippocampal neurones were transfected with EGFP-$C3^{bot}$ or EGFP-$C3^{bot}$ E174A. The intracellular expression of EGFP-$C3^{bot}$ but not of its enzymatically dead form EGFP-$C3^{bot}$ E174A reduced axon length and branching (Fig. 5) comparable to the effects caused by $C3^{lim}$ and $C3^{stau2}$ (see Fig. 3). Expectedly, similar data were obtained by expressing the C3-related EGFP-$C3^{stau2}$ (data not shown). The fact that the enzymatically dead $C3^{bot}$ promotes axonal growth and branching comparable to wild-type $C3^{bot}$, however, without directly inactivating Rho by ADP-ribosylation, supports the notion of a dominant extracellular effect. The negative effects on axon growth observed when inactivating Rho by transfection with $C3^{bot}$ (Fig. 5) or $C3^{stau2}$ (not shown), nicely correlate with the pronounced inhibitory effect of RhoA N19 expression on axon branching and with those effects observed by extracellularly applied $C3^{lim}$ and $C3^{stau2}$ (see Figs 2 and 3b). Thus, at this developmental stage, axonal growth and branching appears to require activation of RhoA.

While RhoA expression promoted axonal development, it inhibited dendritic growth and branching. To substantiate these observations we also analysed C3 effects on dendrites using staining by MAP2 antibody. A total of 20 nM of $C3^{bot}$ as well as 100 nM of either $C3^{lim}$ or $C3^{stau2}$, which all have functional ADP-ribosyltransferase activities and thus Rho-inactivating properties, promoted dendritic length and branching pattern (Figs 6a and b). By contrast, and opposite to the effects on axons, the enzymatically dead C3 was without effect on dendrites (Figs 6a and b). These data indicate that RhoA negatively regulates dendritic growth.

Discussion

The present study provides evidence that axons and dendrites differentially depend on the activation of Rho GTPases, especially RhoA. While axonal growth and branching benefit from active Rho, dendritic growth and branching are inhibited. These conclusions are based on transfection studies using either RhoA constructs or active and inactive C3 isoforms as well as on extracellular application of $C3^{lim}$ or $C3^{stau}$. During the course of this study we became aware that one of the most widely used tools to investigate RhoA effects, $C3^{bot}$, has in addition to its ADP-ribosylating property a novel neurotrophic function not mediated by a direct inactivation of RhoA.

Small GTPases are thought to be involved in morphological changes during neuronal development. These processes are ruled by the genetic-based differentiation mechanisms but are modified by the extracellular matrix (repulsive or not) as well as factors of the surrounding medium or from glial cells

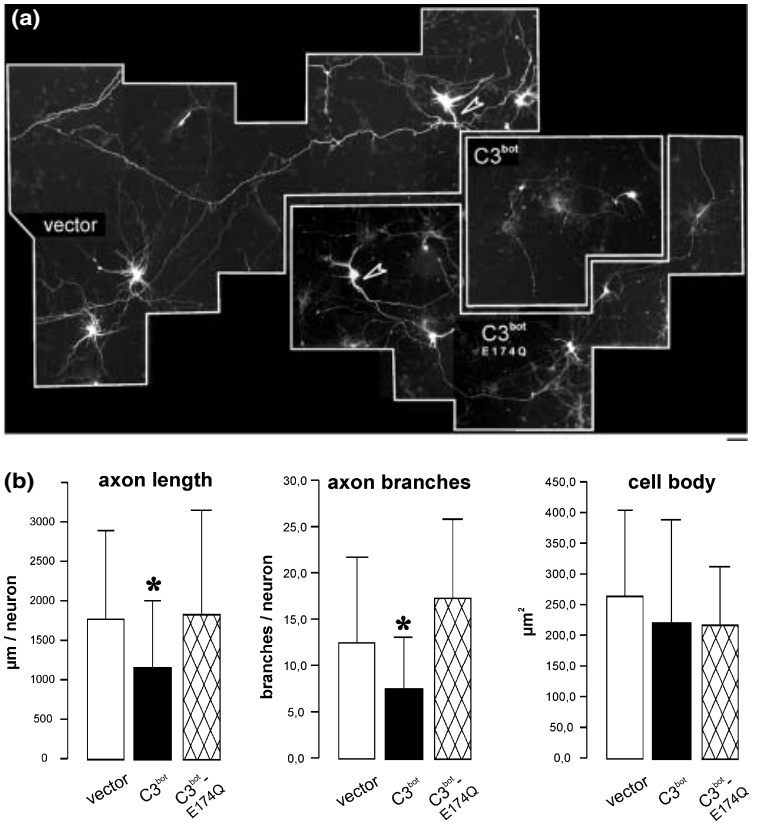

Fig. 5 Exclusively intracellular effects of C3^{bot} expressed in neurones after transfection with pEGFP-vector. C3^{bot} proteins were expressed as fusion proteins with EGFP. Hippocampal neurones (obtained from mice embryos at day 17 and seeded at a density of 2×10^{-5}/well) were transfected with either mere EGFP-vector, EGFP-C3^{bot} or EGFP-C3^{bot} E174Q at DIV4 using the Effectene transfection kit. Neurones were fixed at DIV5 and analysed morphometrically by the Neurolucida software. (a) Representative photomicrographs from either vector-, EGFP-C3^{bot}- or EGFP-C3^{bot} E174Q-transfected neurones are shown. Arrowheads indicate the origins of axon. Scale bar: 50 µm. (b) Morphometric analysis of neurones (obtained in five different preparations) transfected with cDNAs encoding either EGFP alone, EGFP-C3^{bot} or EGFP-C3^{bot} E174Q all three in the same vector. A significant decrease of axon length and branching against vector control (140 neurones) was observed with the ADP-ribosylating EGFP-C3^{bot} (100 neurones; $p = 2.69E\text{-}4$ and 7.4E-6 for axon length or branching, respectively), whereas transfection with the enzymatically dead EGFP-C3^{bot} E174Q (98 neurones) had no effect on the morphology of the neurones. For each transfection condition the size of the cell bodies were comparable. Note that C3^{bot} when acting exclusively intracellularly inhibits axonal growth, whereas C3^{bot} E174Q does not.

like S100β (Azmitia *et al*. 1990) or ADNF (activity-dependent neurotrophic factor; Blondel *et al*. 2000). Furthermore, the effects of Rho are crucially dependent on the stage of differentiation at which Rho is activated, a factor that probably explains many of the conflicting data in the literature. For instance, RhoA has been suggested to either inhibit neurite formation (Jin and Strittmatter 1997; Lehmann *et al*. 1999; Yamashita *et al*. 1999; Bito *et al*. 2000; Dergham *et al*. 2002) or to promote axonal and dendritic growth (Threadgill *et al*. 1997). Most of these studies have analysed early developmental stages and used serum which contains growth-promoting and inhibitory factors.

A second problem relates to the fact that many studies addressing the role of Rho activation in neuronal differentiation rely on C3^{bot} as a specific tool for activating RhoA, -B or -C. Our surprising finding that C3^{bot} promotes axonal growth and branching independent of its enzymatic activity shows that this toxin cannot be regarded anymore as a specific Rho inactivator unless it is established that the activity is dependent on its ADP-ribosylating activity.

At developmentally advanced (i.e. stage 4) hippocampal neurones cultivated in serum-free medium, RhoA exhibits opposite effects on axonal and dendritic growth and branching (this paper). When testing the *in vivo* function of Rho in *Drosophila* using loss-of-function mutants it turned out that at least RhoA is not necessary for correct axon outgrowth, guidance, branch formation or synaptic vesicle distribution, whereas it appears to inhibit dendritic growth and branching (Lee *et al*. 2000). Our data also suggest that, at least at a certain developmental stage, RhoA is supportive for axon growth and branching and negatively regulates dendritic growth and branching. Most of the data showing a negative

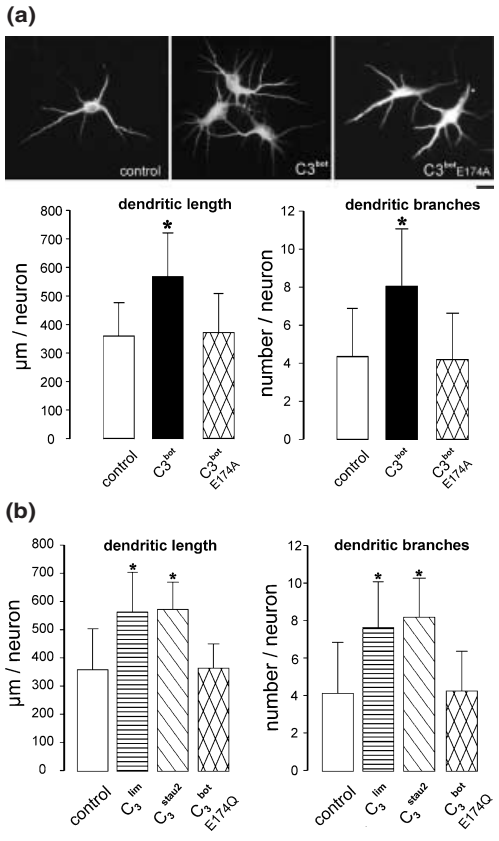

Fig. 6 Effects of C3 isoforms on dendritic growth of hippocampal neurones. (a) The indicated $C3^{bot}$ isoforms were applied at DIV1 to hippocampal neurones at a concentration of 20 nM for 5 days. Neurones were stained with an antibody against the microtubule-associated protein 2 (MAP2) and photomicrographs (upper panel) from individual neurones were subjected to morphometrical analysis using Neurolucida software. The length of all dendritic processes (left diagram) and the number of all primary and secondary dendrites (right diagram) were quantified. The following *p*-values calculated against controls (56 neurones from three preparations) were obtained: dendritic length: $C3^{bot}$ (60 neurones from three preparations): 1.9E-12; $C3^{bot}$ E174A (62 neurones from three preparations): 0.7 and for dendritic branches $C3^{bot}$ 4.2E-10. Note that only $C3^{bot}$ but not $C3^{bot}$ E174A promote dendritic growth. Scale bar: 20 µm. (b) $C3^{lim}$, $C3^{stau2}$ and $C3^{bot}$ E174Q were applied at DIV1 to hippocampal neurones at a concentration of 100 nM for 5 days. Neurones were stained with an antibody against the microtubule-associated protein 2 (MAP2) and photomicrographs from individual neurones were subjected to morphometrical analysis using Neurolucida software. The length of all dendritic processes (left diagram) and the number of all primary and secondary dendrites (right diagram) were quantified. The following *p*-values against controls (23 neurones) were obtained: dendritic length: 0.0021, $C3^{lim}$ (21 neurones); 0.003, $C3^{stau2}$ (19 neurones) and for dendritic branches 0.001, $C3^{lim}$; 0.0005, $C3^{stau2}$, whereas no signficant effect was obtained after treatment with $C3^{bot}$ E174Q (27 neurones). Note that only the enzymatically active C3-proteins but not the enzymatically dead $C3^{bot}$ E174Q affected dendritic growth.

effect of RhoA on neurite or axon growth used substrates like myelin, chondroitin sulphate proteoglycane (CSPG) or aggrecan, which are avoided by growth cones and outgrowing axons (Lehmann *et al*. 1999; Borisoff *et al*. 2003; Monnier *et al*. 2003). So, it may be that RhoA mediates a specific avoidance behaviour which can be overcome by introducing C3 proteins, $C3^{bot}$ (Dergham *et al*. 2002) or $C3^{lim}$ (Monnier *et al*. 2003). In contrast, axon growth and branching in general may benefit from active RhoA supported by our transfections studies and probably also from RhoB or -C, all three of which are inactivated by intracellularly expressed $C3^{bot}$ following transfection. At least for axon growth, defects in RhoA may be overcome during development as seen in the deletion mutants from drosophila (Lee *et al*. 2000).

A variety of experiments including gene deletion of Rac and RhoA in *Drosophila* suggest that different levels of especially Rac activation instruct either axon growth, branching or guidance (for review see Luo 2002). Similarly, variable levels of activated Rho may be differentially linked to these three morphological changes with RhoA being preferentially involved in axonal growth avoidance on special substrates (see above) and also in axonal branching at developmentally advanced stages (this paper). In this respect, RhoA-dependent effects may also vary when comparing subpopulations of neurones. For cerebellar neurones, different amounts of RhoA appear to address different signal transduction pathways resulting either in negative (ROCK-mediated) or positive (mDia1-mediated) effects on early axon growth (Arakawa *et al*. 2003). At least for embryonic hippocampus in culture, our study shows that the RhoA-dependent effects on axons appear to apply to all neurones.

Dendritic growth and branching, by contrast, are inhibited by active RhoA and benefit when Rho proteins are inactivated by C3-catalysed ADP-ribosylation.

Transfected constitutive active RhoA negatively influences dendritic growth and branching, whereas the dominant negative RhoA has promoting effects. This notion is supported by the fact that all enzymatically active C3 proteins have dendritic-promoting effects when applied extracellularly. In line with this finding, negative effects of RhoA on dendritic growth are also described in *Drosophila* (Lee *et al*. 2000) and hippocampal slice cultures (Nakayama *et al*. 2000).

Thus, it appears that Rho proteins are involved in all steps during the developmental differentiation of neurones. Activated RhoA, probably in close cross-talk with other Rho proteins like Rac, causes the collapse of growth cones, promotes the growth and branching of established axons and add to correct guidance by causing retraction from unfavourable substrates. For dendrites, less is known about the regulation of their growth cones and guidance, but activated Rho proteins appear to inhibit growth and branching *in vivo*

(Lee *et al*. 2000) as well as under culture conditions (this paper).

To date, neurotrophic-like effects of C3 proteins have been exclusively linked to an inactivation of Rho, mainly RhoA. $C3^{bot}$, and most notably the enzymatically dead $C3^{bot}$ E174A and $C3^{bot}$ E174Q, promote axon growth when applied at nanomolar concentrations. Their neurotrophic effects are definitely not mediated by a direct inhibition of Rho GTPases. The low concentrations required to elicit axonotrophic effects may suggest an interaction with a neurotrophic factor receptor. Even more, axon growth-promoting effects are not shared when using the related proteins $C3^{lim}$ and $C3^{stau2}$. The specificity of $C3^{bot}$ is further underlined by the fact that intracellularly expressed $C3^{bot}$ harbouring functional ADP-ribosyltransferase activity exerts opposing effects on axonal growth and branching compared to the extracellularly applied. Thus, the axon-promoting effects of $C3^{bot}$ appear to be dominant over its effects on inactivating RhoA, -B or -C. $C3^{bot}$ applied extracellularly has Rho-independent or -dependent effects on either axon and dendrite morphology, respectively. In both cases, binding to a high-affinity binding site appears to be the first step as effects are discernible at the nanomolar range. At least the effects on dendrites require an internalization step, because they are based on the inactivation of Rho. So, it may be speculated that for both enzymatically active and dead $C3^{bot}$ binding as well as internalization are mediated by the same membrane structure. It is also tentative to assume that the axon-promoting effects of $C3^{bot}$, $C3^{bot}$ E174A, or $C3^{bot}$ E174Q, mediated either by an as yet unidentified receptor or by an intracellular protein interaction, may change the activity state of one of the Rho GTPases.

Taken together, axonal and dendritic growth are differently regulated by RhoA GTPase, whereby RhoA is repressive for dendritic but permissive for axonal growth. This concept is based on expression studies with constitutive active and dominant negative RhoA forms as well as the application of the 'Rho inhibitor' C3. Furthermore, this study unraveled that the family of C3-like ADP-ribosyltrans-ferases – widely used as cell biological tools – is not a uniform entity but that especially the $C3^{bot}$ isoform possesses an additional ligand-like neurotrophic effect which is clearly different from is enzymatic activity.

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