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EDITOR'S CHOICE

Serum-induced neurite retraction in CAD cells – involvement of an ATP-actin retractile system and the lack of microtubule-associated proteins

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Cultured catecholamine-differentiated cells [which lack the microtubule-associated proteins (MAPs): MAP1B, MAP2, Tau, STOP, and Doublecortin] proliferate in the presence of fetal bovine serum, and, in its absence, cease dividing and generate processes similar to the neurites of normal neurons. The reintroduction of serum induces neurite retraction, and proliferation resumes. The neurite retraction process in catecholamine-differentiated cells was partially characterized in this study. Microtubules in the cells were found to be in a highly dynamic state, and tubulin in the microtubules consisted primarily of the tyrosinated and deacetylated isotypes. Increased levels of acetylated or Δ2-tubulin (which are normally absent) did not prevent serum-induced neurite retraction. Treatment of differentiated cells with lysophosphatidic acid or adenosine deaminase induced neurite retraction. Inhibition of Rho-associated protein kinase, ATP depletion and microfilament disruption each (individually) blocked serum-induced neurite retraction, suggesting that an ATP-dependent actomyosin system underlies the mechanism of neurite retraction. Nocodazole treatment induced neurite retraction, but this effect was blocked by pretreatment with the microtubule-stabilizing drug paclitaxel (Taxol). Paclitaxel did not prevent serum-induced or lysophosphatidic acid-induced retraction, suggesting that integrity of microtubules (despite their dynamic state) is necessary to maintain neurite elongation, and that paclitaxel-induced stabilization alone is not sufficient to resist the retraction force induced by serum. Transfection with green fluorescent protein–Tau conferred resistance to retraction caused by serum. We hypothesize that, in normal neurons (cultured or *in vivo*), MAPs are necessary not only to stabilize microtubules, but also to establish interactions with other cytoskeletal or membrane components to form a stable structure capable of resisting the retraction force.

Introduction

There have been few studies on the process of neurite retraction in comparison with the number of studies on elongation, guidance and synapses of neural processes. Because of the great complexity of the nervous system,

Abbreviations
Ac-Tub, acetylated tubulin; ADA, adenosine deaminase; CAD, catecholamine-differentiated; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; Glu-Tub, detyrosinated tubulin; LPA, lysophosphatidic acid; MAP, microtubule-associated protein; MT, microtubule; ROCK, Rho-associated protein kinase; TSA, trichostatin A; Tyr-Tub, tyrosinated tubulin.

researchers tend to first investigate how neurons are built. The use of cultured neurons provides a useful tool for this purpose. In spite of the successful primary culture of certain types of neurons, it is difficult to overcome certain methodological problems, including the time-consuming and delicate procedures required for preparation of cells, and the limited numbers of cells that can be obtained. Certain types of cultured neuron-like cells have been successfully utilized in many studies. CAD (Cath.a-differentiated) cells are derived from a catecholaminergic central nervous system cell line established by targeted oncogenesis in transgenic mice. These cells provide a useful model for studies of neurite elongation and retraction because: (a) the cells are rounded and proliferate in standard culture medium containing fetal bovine serum; (b) when deprived of fetal bovine serum, the cells stop proliferating and generate long processes that are similar in many respects to those of normal neurons; and (c) when fetal bovine serum is reintroduced, the processes rapidly retract and the cells resume growth. CAD cells express neuron-specific proteins such as class III β-tubulin, GAP-43, SNAP-25, synaptotagmin, and other neuropeptides [1,2]. The intracellular transport system powered by kinesins and dyneins functions similarly to other neuronal systems [3–5]. Electrophysiological studies have demonstrated that action potentials can be induced and voltage-dependent sodium and potassium currents can be elicited in CAD cells [6]. The microfilament and neurofilament systems appear to be normal. Several microtubule (MT)-associated proteins (MAPs: MAP1B, MAP2, Tau, STOP, and Doublecortin) are absent in differentiated and undifferentiated CAD cells [7].

MTs are highly dynamic structures formed predominantly by tyrosinated tubulin (Tyr-Tub); the detyrosinated isotype (Glu-Tub) is scarce, Δ2-tubulin is absent, and the acetylated isotype (Ac-Tub) is essentially undetectable. In spite of the absence of stabilizing proteins and stabilized MTs, the rate of neurite elongation of CAD cells is similar to that of cultured rat hippocampal neurons [7]. We found that neurite retraction in CAD cells is sensitive to lysophosphatidic acid (LPA) and adenosine deaminase (ADA), and that an ATP-hydrolyzing actomyosin system is responsible for the centripetal force that leads to neurite retraction in response to fetal bovine serum addition. The numerous properties and events that are similar to those of normal cells, and the unusual absence of MAPs, make CAD cells an excellent system for studies of neuronal functions in which MAPs are suspected to be involved. The results of the present study suggest that MAPs in primary cultured neurons function not only to stabilize MTs, but also to establish contacts with other cellular components, leading to the formation of a structure resistant to the contractile force of the actomyosin system that tends to retract neurites.

Results

Neurite retraction induced by LPA and by ADA

The identities and properties of the compounds in fetal bovine serum that elicit neurite retraction in CAD cells are of obvious interest. Several compounds other than fetal bovine serum have been found to induce neurite retraction in various cells lines, e.g. thrombin, LPA, prostaglandins, dopamine, propofol, and nitric oxide [8–12]. We examined the abilities of some of these compounds to induce neurite retraction in CAD cells.

LPA is a phospholipid that serves as a precursor in *de novo* lipid synthesis and produces many cellular responses through its interaction with G-protein-coupled transmembrane LPA receptors. It is involved in a variety of cellular phenomena, including cytoskeletal actin rearrangement leading to neurite collapse or axon branching [13,14], stimulation or inhibition of cell proliferation [15], and inhibition of gap junction communication [16]. In the present study, treatment of cells with purified LPA resulted in neurite retraction similar to that induced by fetal bovine serum (Fig. 1A, B). This finding suggests that neurite retraction in CAD cells is governed by signaling cascades that are common to various types of neurons and neuronal cell lines [11,17–19].

ADA is an enzyme that catalyzes the hydrolytic deamination of adenosine to inosine. The location of ADA is mainly cytoplasmic, but it is also found associated with membrane fractions [20,21]. In addition to its primary function, ADA has been shown to stimulate the release of excitatory amino acids through a mechanism independent of adenosine depletion [22]. In the same study, ADA was shown to induce calcium influx and increased phosphoinositide hydrolysis. In view of the finding by Tigyi *et al*. (1996) [11] that calcium and phosphoinositides are involved in neurite retraction, we hypothesized that ADA could have a retracting effect in our experimental model. Treatment of differentiated CAD cells with 10 $U \cdot mL^{-1}$ ADA resulted in partial neurite retraction (Fig. 1A,B).

The tyrosination and acetylation state of tubulin in relation to MT dynamics in CAD cells

Two of the most extensively studied post-translational modifications of tubulin are the cyclic detyrosination/tyrosination that occurs at the C-terminus of the

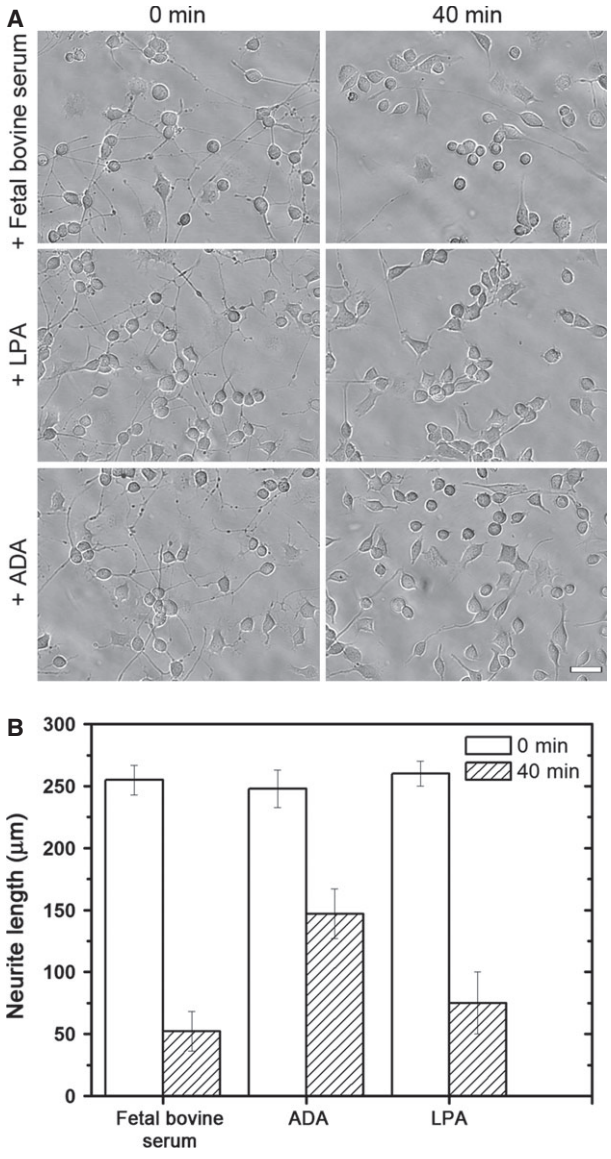

Fig. 1. Effects of fetal bovine serum, LPA and ADA treatment on neurite length. (A) CAD cells that had differentiated for 5 days were treated with purified ADA (final concentration of 10 U·mL^{-1}), purified LPA (final concentration of 2 μM), or fetal bovine serum (final concentration of 10%). Images were taken at $t = 0$ and 40 min. Scale bar: 45 μm. (B) Quantification of neurite length at $t = 0$ and 40 min. The values shown are mean ± standard deviation from three independent experiments.

α-chain and the acetylation/deacetylation of the ε-NH_2-group of lysine at position 40 of the α-chain. These modifications in cells determine various combinations of tubulins and MTs based on their state of tyrosination (±) and acetylation (±). High levels of Glu-Tub or Ac-Tub are frequently used as 'markers' of stable MTs, based on previously determined kinetics of the two reactions [23–25]. In contrast, high levels of Tyr-Tub or nonacetylated tubulin are indicative of dynamic MTs. Our fluorescence recovery after photobleaching (FRAP) analysis of MT dynamics in differentiated cells showed that these structures are highly dynamic and have a half-life of 5.5 min (Fig. 2B). Measurement of sensitivity to nocodazole treatment indicated a half-life of 6 min (Fig. 3C).

Detyrosination and acetylation of tubulin are generally considered to be consequences rather than causes of MT stabilization; however, some uncertainty regarding this relationship remains. In CAD cells, Tyr-Tub is the predominant tubulin isotype, the level of Glu-Tub is very low, Δ2-tubulin is absent, and Ac-Tub is essentially undetectable (Fig. 3A). These

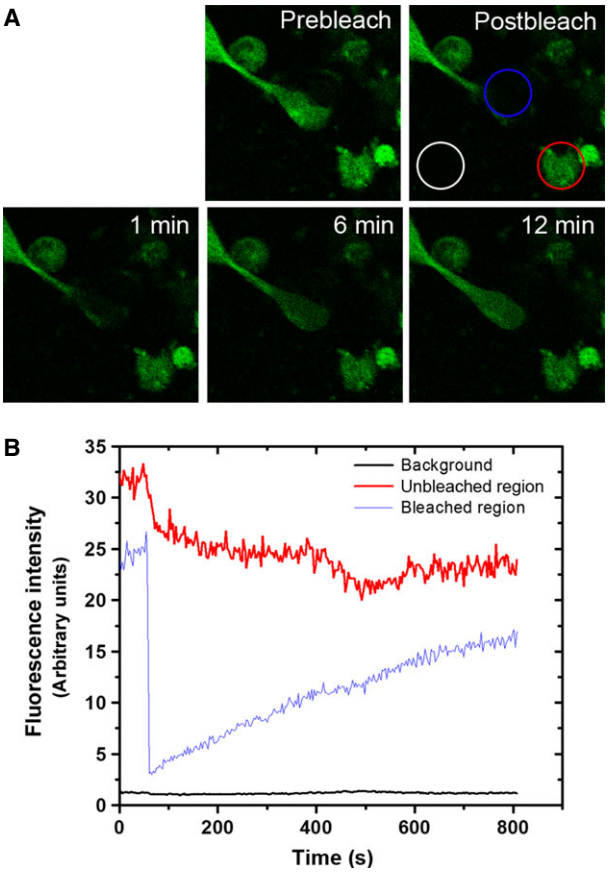

Fig. 2. Dynamics of MTs of differentiated CAD cells as assessed by FRAP. (A) Undifferentiated cells were transfected with GFP–Tyr-Tub, and cultured for 48 h under differentiating conditions; appropriate neurites were then selected for FRAP analysis. Blue circle: bleached region. White circle: background. Red circle: unbleached region. Images were taken every 3 s during a 14-min period after bleaching. Images at the indicated times are from a typical experiment from two replicates. (B) Quantification of fluorescence intensity of the three regions in (A).

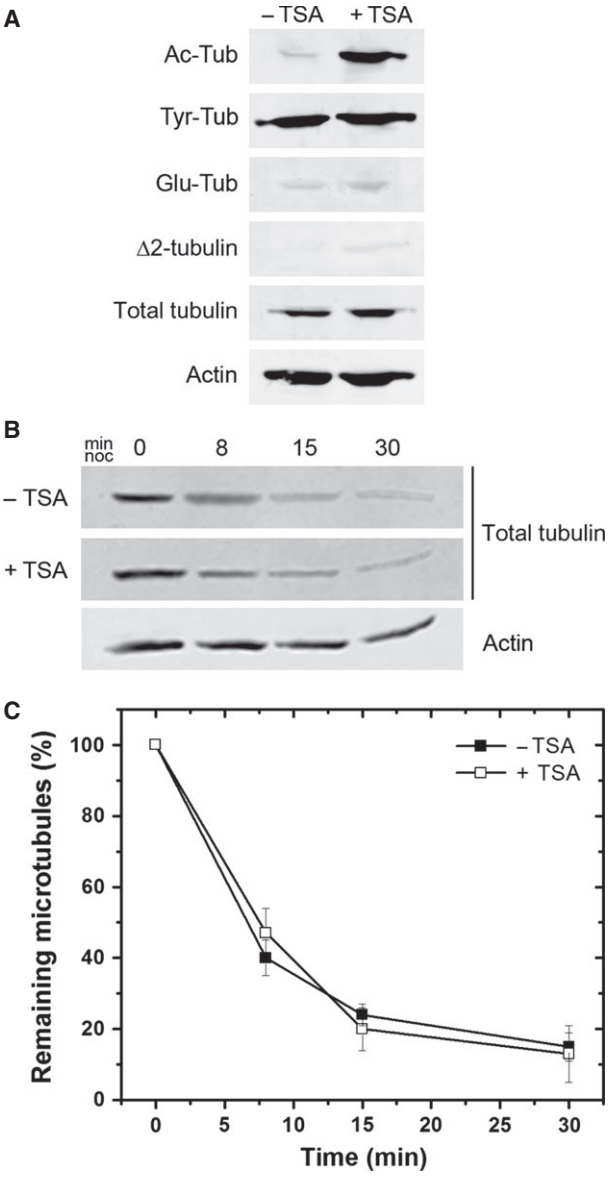

Fig. 3. Acetylation and tyrosination state of tubulin in relation to MT dynamics. (A) CAD cells were treated (+) or not treated (–) with 0.5 μM TSA, dissolved in Laemmli sample buffer, for 16 h, and subjected to western blotting and staining of separate aliquots by the indicated antibodies. (B) Cells that had differentiated for 5 days were treated (+) or not treated (–) with 0.5 μM TSA for 16 h in culture medium lacking fetal bovine serum, incubated with 10 μM nocodazole (noc) for the indicated times, and immediately processed for isolation of the cytoskeletal fraction (i.e. MTs remaining after nocodazole treatment), as described in Experimental procedures. This fraction was subjected to western blotting with mAb DM1A. (C) Quantification of remaining MTs as a function of time. The optical density corresponding to the tubulin bands in the western blots shown in (B) were measured and expressed as a percentage of the value at $t = 0$. The values shown are mean ± standard deviation from three independent experiments.

observations of tyrosination and acetylation states are consistent with the highly dynamic state of MTs (Figs 2B and 3C). In view of the relationship between Ac-Tub and stable MTs, we examined the effect of a transient increase in the level of Ac-Tub on MT dynamics and on neurite retraction induced by fetal bovine serum-containing medium. An increase in the level of Ac-Tub caused by trichostatin A (TSA) treatment did not modify MT dynamics in neurites, as assessed by sensitivity to nocodazole (Fig. 3B,C). Similarly, TSA treatment had no effect on fetal bovine serum-induced neurite retraction (Fig. 4A,B). The transient increase in the level of Ac-Tub caused by TSA treatment had no significant effect on the tubulin tyrosination state (Fig. 3A).

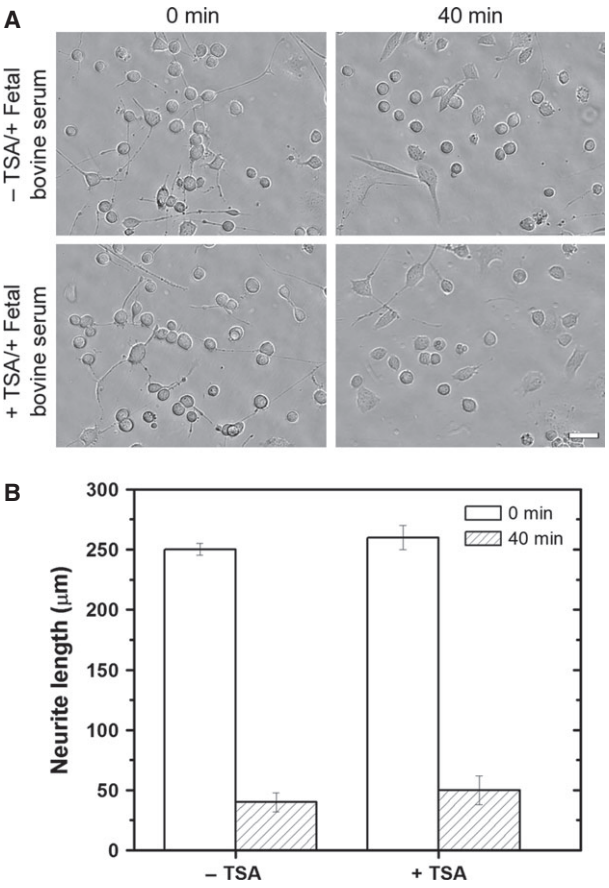

Fig. 4. Effect of TSA treatment on fetal bovine serum-induced neurite retraction. (A) CAD cells that had differentiated for 5 days were treated (+) or not treated (–) with 0.5 μM TSA for 16 h, and fetal bovine serum was added to the medium (final concentration of 10%). The images show representative fields of the cells prior to fetal bovine serum addition (0 min) and after 40 min of incubation. Scale bar: 45 μm. (B) Quantification of neurite length before (0 min) and after (40 min) fetal bovine serum addition. The values shown are mean ± standard deviation from four independent experiments.

The Δ2-tubulin isotype is easily detectable in brain tissue, being present in differentiated neurons, and abundant in long-lived animal species [26–28]. Mature brain neurons and long-lived neurons in culture contain a high proportion of stable MTs, suggesting that Δ2-tubulin may be involved in MT stabilization and impede neurite retraction. To test this possibility, we transfected CAD cells with the green fluorescent protein (GFP)–Δ2-α-tubulin plasmid (which lacks sequences corresponding to the last two C-terminal amino acids), and examined the effect of such transfection on fetal bovine serum-induced neurite retraction. Neurites generated by the transfected cells showed retraction behavior similar to that of control cells transfected with GFP–Tyr-α-tubulin plasmid (Fig. 5).

These findings indicate that the transient expression of tubulin isotypes associated with stable MTs does not generate neurite stabilization when retraction is induced by fetal bovine serum.

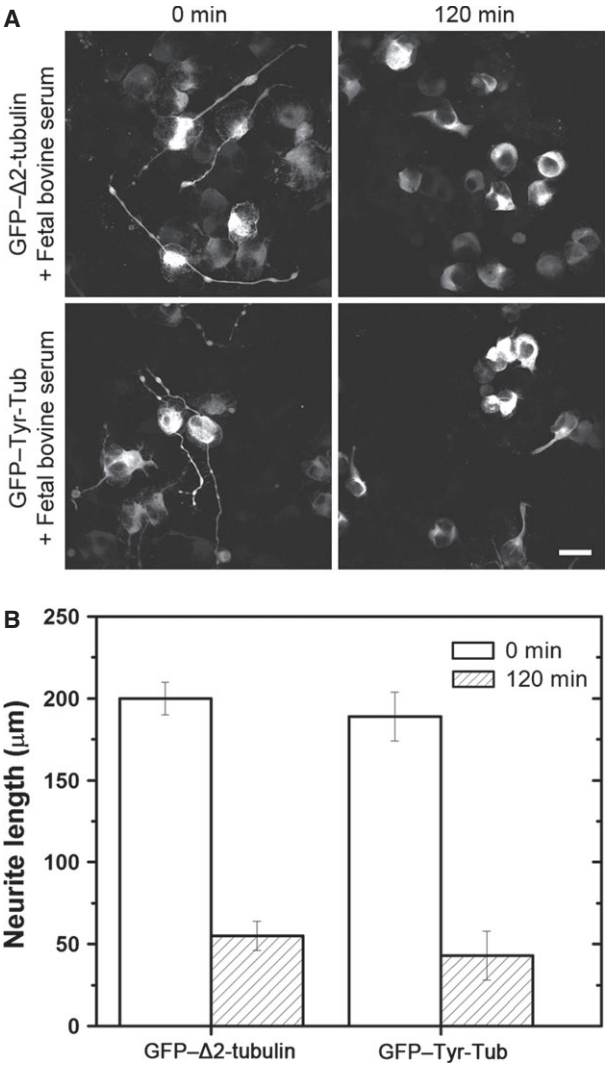

Fig. 5. Effect of Δ2-tubulin transfection on fetal bovine serum-induced neurite retraction. (A) Undifferentiated CAD cells were transfected with plasmids carrying GFP–Δ2-tubulin or GFP–Tyr-Tub. After 48 h under differentiating conditions, cells were tested for fetal bovine serum-induced neurite retraction. Cells were fixed before ($t = 0$) or after (120 min) serum addition. The photographs show fluorescent images from a typical experiment. Scale bar: 25 μm. (B) Quantification of neurite lengths of transfected cells before and after fetal bovine serum addition. For statistical analysis of neurite length for each sample, five different fields were analyzed per experiment. The values shown are mean ± standard deviation from three independent experiments.

Neurite retraction is independent of MT dynamics

Neural processes retract rather than undergoing further elongation if an MT-depolymerizing drug (e.g. nocodazole) is added to the culture medium [11,29]. In the present study, serial photographs taken during CAD cell differentiation in fetal bovine serum-free medium showed that elongation proceeded at rate similar to that of cultured hippocampal rat neurons (data not shown). Upon addition of nocodazole (final concentration of 10 μM) to the culture medium, the neurites stopped elongating and began to retract. The neurites showed ~ 50% retraction 40 min after nocodazole addition (Fig. 6B), in comparison with ~ 80% retraction induced by fetal bovine serum addition. These two types of neurite retraction were similar but not identical. Nocodazole treatment resulted in decreased neurite diameters (Fig. 6A, insets) and subsequent fragmentation and/or disappearance, leading to neurite shortening. In contrast, fetal bovine serum-induced neurite retraction involved a decrease in neurite length without a notable reduction in diameter or fragmentation (Fig. 6A, insets).

Pretreatment of differentiated CAD cells with the MT-stabilizing drug paclitaxel (Taxol) interfered somehow with fetal bovine serum-induced neurite retraction. A few minutes after fetal bovine serum addition, we observed abnormal retraction involving reduced diameters and fragmentation of neurites (data not shown), leading to neurite shortening after ~ 40 min. The same phenomenon was observed when LPA was added to paclitaxel-pretreated cells. In contrast, pretreatment of cells with paclitaxel and subsequent treatment with nocodazole did not result in neurite retraction (Fig. 7A,B). These findings suggest that different biochemical mechanisms control nocodazole-induced and fetal bovine serum/LPA-induced neurite retraction.

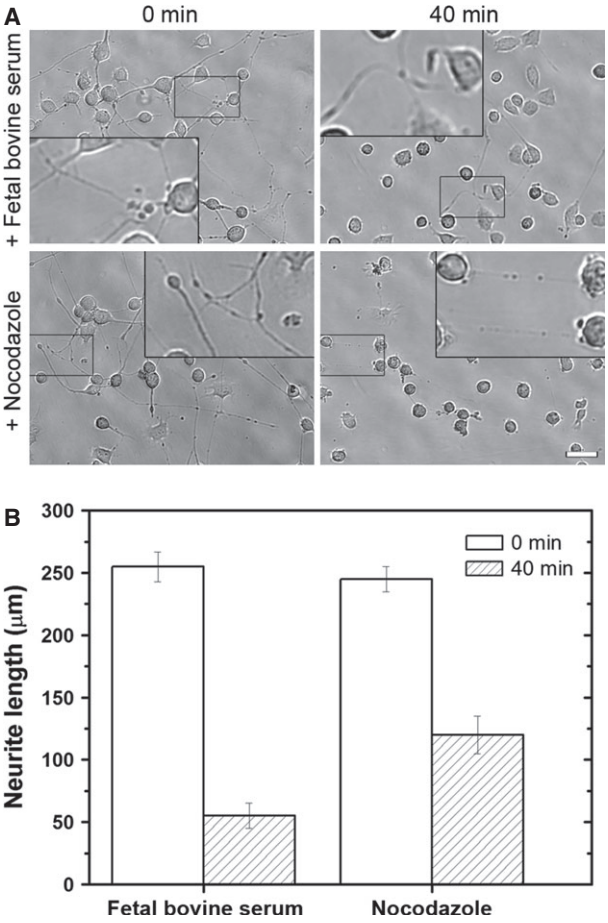

Fig. 6. Comparison of fetal bovine serum-induced and nocodazole-induced neurite retraction. (A) CAD cells that had differentiated for 5 days were treated with 10 μM nocodazole or 10% fetal bovine serum. For each sample, photographs of several fields were taken at $t = 0$ and 40 min. Images from a typical experiment are shown. Scale bar: 45 μm. (B) Quantification of neurite length at $t = 0$ and 40 min. The values shown are mean ± standard deviation from three independent experiments.

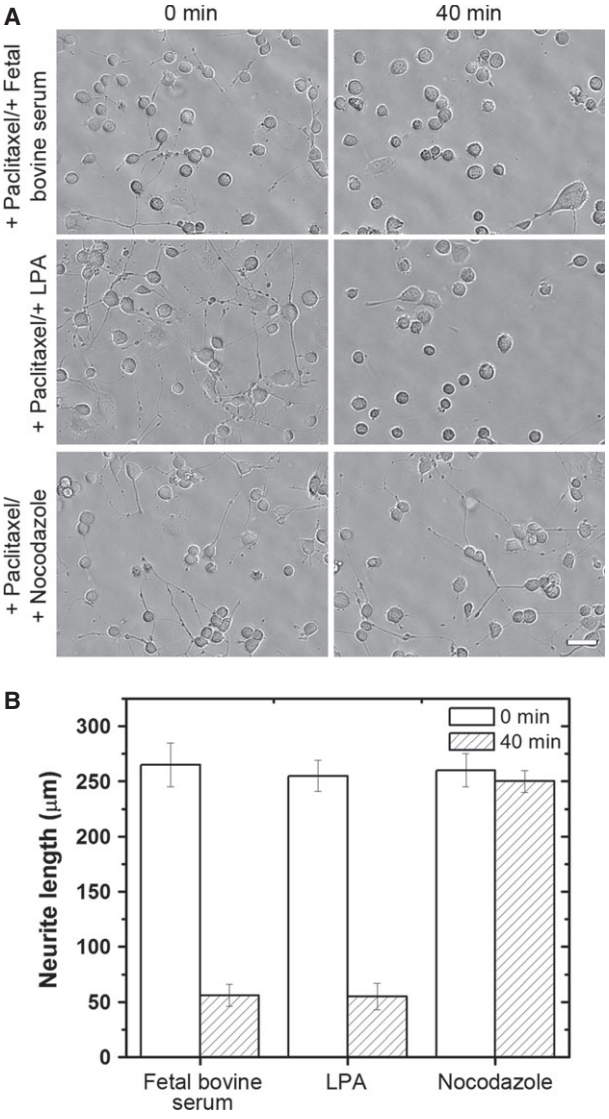

Fig. 7. Effect of pretreatment with paclitaxel on fetal bovine serum-induced, LPA-induced and nocodazole-induced neurite retraction. (A) CAD cells that had differentiated for 5 days were pretreated for 2 h with 10 μM paclitaxel, and then treated with 10% fetal bovine serum, 2 μM LPA, or 10 μM nocodazole. Images were taken at $t = 0$ and 40 min. Scale bar: 45 μm. (B) Quantification of neurite length at $t = 0$ and 40 min. The values shown are mean ± standard deviation of four independent experiments.

An ATP-dependent actomyosin system is involved in fetal bovine serum-induced neurite retraction

When differentiated CAD cells were treated with sodium azide to decrease the level of intracellular ATP, subsequent addition of fetal bovine serum to the culture medium did not result in complete neurite retraction (Fig. 8A). Similarly, after treatment of differentiated cells with cytochalasin D to disrupt microfilament structure, fetal bovine serum addition did not result in neurite retraction (Fig. 8B). These findings suggest that the integrity of the filamentous actomyosin system operating in an ATP-dependent mechanism is essential for fetal bovine serum-induced neurite retraction in CAD cells. Treatment of differentiated cells with the Rho-associated protein kinase (ROCK) inhibitor Y-27632 also inhibited fetal bovine serum-induced neurite retraction (Fig. 9A,B), suggesting that ROCK and microfilaments are involved in the generation of actomyosin contractility through Rho kinase-dependent signaling. This pathway is presumably responsible for the generation of the contractile force

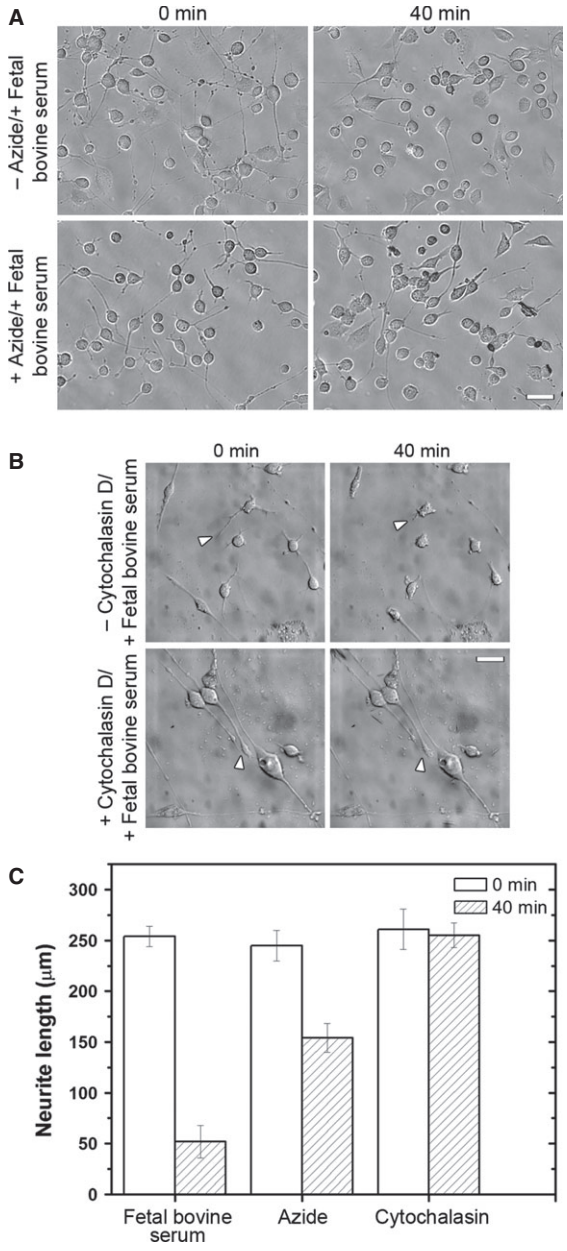

Fig. 8. Effects of intracellular ATP depletion and microfilament disruption on fetal bovine serum-induced neurite retraction. (A) CAD cells that had differentiated for 5 days were cultured for 30 min in NaCl/P_i in the presence (+) or absence (–) of 20 mM sodium azide, and fetal bovine serum (final concentration of 10%) was added to the culture medium. Images were taken at $t = 0$ and 40 min after fetal bovine serum addition. Scale bar: 45 μm. (B) Cells that had differentiated for 5 days were cultured for 10 min in the presence (+) or absence (–) of 60 μM cytochalasin D, and fetal bovine serum (final concentration of 10%) was added to the culture medium. Images of the same field were taken at $t = 0$ and 40 min after fetal bovine serum addition. Scale bar: 45 μm. (C) Quantification of neurite length at $t = 0$ and 40 min, from five different fields per experiment. The values shown are mean ± standard deviation from three independent experiments.

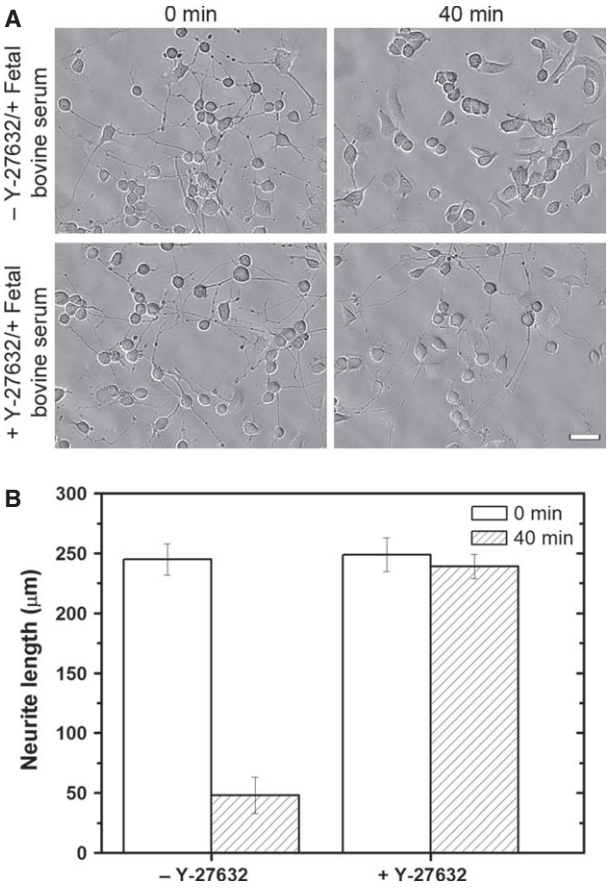

Fig. 9. Effect of ROCK inhibition on fetal bovine serum-induced neurite retraction. (A) CAD cells that had differentiated for 5 days were cultured for 15 min in the presence (+) or absence (–) of 10 μM Y-27632, and fetal bovine serum (final concentration of 10%) was added to the culture medium. Images were taken at $t = 0$ and 40 min after fetal bovine serum addition. Scale bar: 45 μm. (B) Quantification of neurite length at $t = 0$ and 40 min. The values are mean ± standard deviation from three independent experiments.

that produces fetal bovine serum-induced neurite retraction in CAD cells.

Transfection of CAD cells with GFP–Tau induces resistance to retraction of neural processes

Taking into account that the development of healthy processes in cultures of cells of neural origin requires the presence of serum in the medium, an intriguing question arising from this work is why processes generated by CAD cells retract when serum or other factors are included in the culture medium. We considered the possibility that this was attributable to the reported lack of MAPs. We therefore transfected CAD cells with a plasmid containing GFP–Tau, and,

after 2–3 days of differentiation, added serum and determined the presence of processes at time zero ($t = 0$, immediately before serum addition) and after 15 h. At the corresponding times, cells were fixed and subjected to immunofluorescence with the DM1A antibody against α-tubulin to visualize all of the processes. Figure 10A shows that, at $t = 0$, most of the processes (tubulin in red) were not positive for GFP–Tau. However, after 15 h in the presence of serum, most of the processes that had resisted retraction were GFP–Tau-positive. Approximately 20 fields from three independent experiments were analyzed for quantitative determination of red and green processes. The results from the quantitative analysis in Fig. 10B show that, at $t = 0$, 32% of the total processes expressed GFP–Tau, whereas after 15 h of retraction 88% of the processes that resisted the retraction contained GFP–Tau. This result indicates that practically all of the processes lacking GFP–Tau were retracted during the 15-h period, whereas those in which Tau was expressed were not sensitive to the retractile action of serum. In other words, even though we do not know the detailed mechanism, it seems that Tau confers resistance to the retraction induced by serum on CAD processes. This is also supported by the fact that nontransfected CAD cells that were differentiated for 2 days had practically no processes after 15 h in the presence of serum, whereas 20% of the GFP–Tau-transfected cells cultured in parallel had one or more processes (result not shown).

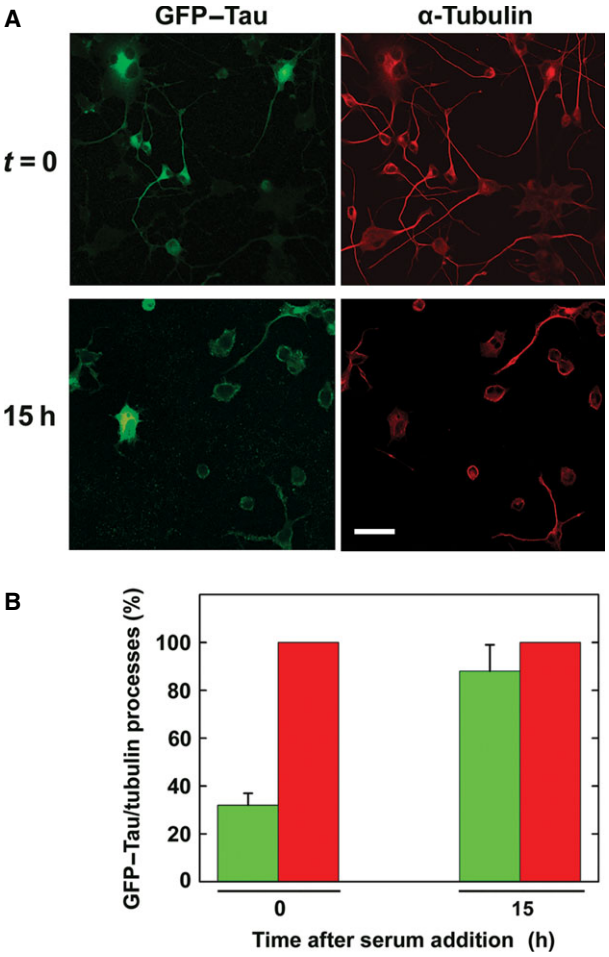

Fig. 10. Expression of GFP–Tau in CAD cells confers resistance to the retraction of the processes. (A) Representative confocal images of CAD cells transfected with the PcDNA3-GFP–Tau23 plasmid as described in Experimental procedures, and differentiated for 2–3 days. Images correspond to samples that were stained with antibody against α-tubulin processed for immunofluorescence before ($t = 0$) and 15 h after the addition of serum to induce retraction of the processes. Photographs from different fields of several samples were taken with a Confocal Spectral version FLOWVIEW 1000 (Olympus) in the green and red channels. Scale bar: 45 μm. Because the amount of GFP–Tau bound to MTs is low in comparison with tubulin, green processes are difficult to visualize. We therefore slightly increased the contrast of images in the green channel. (B) The numbers of processes containing GFP–Tau (green) and MTs (red) were measured in at least 10 fields of different samples. Only processes longer than 40 μm were considered for counting. For samples at $t = 0$ and 15 h, the percentages of processes expressing GFP–Tau (green) were calculated with respect to total processes (100%). In each case, total processes were considered to be the number of processes stained with the DM1A antibody. Values are the mean ± standard deviation from three independent experiments.

Discussion

We found that neurites of cultured CAD cells retract under a centripetal force generated by an ATP-dependent actomyosin system (Fig. 8), which is similar to findings in other types of neural cells. This force-generating system is activated by factors present in fetal bovine serum. A question thus arises regarding the mechanism whereby neurons resist neurite retraction or even show neurite elongation under conditions in which retraction inducers are present, e.g. *in vivo* systems and primary cultures of neurons. We demonstrated previously that CAD cells lack several of the major MAPs, and that MTs are highly dynamic under the above conditions [7]. It is therefore possible that retraction in normal cells is not effective even in the presence of retraction inducers, because MTs are stabilized by a single MAP or the joint action of several MAPs. This concept is consistent with the generally accepted idea that MT stabilization is essential for normal elongation and functioning of neuronal processes [30–32].

The distinctive characteristic of CAD cells of lacking several MAPs allowed us to study the effect of MT stabilization on neurite retraction induced by fetal bovine serum or LPA. MT stabilization alone was not sufficient to impede neurite retraction generated by fetal bovine serum or LPA (Fig. 7). We therefore examined the cells for other differences from normal neurons that may be responsible for neurite retraction even when MTs are stabilized with paclitaxel. As observed here and in our previous study [7], the primary tubulin component of CAD cells is Tyr-Tub; Glu-Tub is scarce, Δ2-tubulin is absent, and the Ac-Tub content is negligible. Mature brain neurons contain many stable MTs and a high Δ2-tubulin content, suggesting a relationship between this tubulin isotype and stabilization. The acetylation of tubulin has been considered to be a marker of 'stable MTs'. However, neither a pharmacological increase in Ac-Tub content nor transfection with Δ2-tubulin impeded neurite retraction in CAD cells (Figs 4 and 5).

We were unable to find differences between CAD cells and normal neurons other than those already described. We therefore focused on the major known difference: the absence of MAPs. We were able to study the effect of MT stabilization in the absence of MAPs in these cells. Because MT stabilization alone (by paclitaxel) was not sufficient to impede retraction, we hypothesize that MAPs in normal neurons, in addition to their role in MT stabilization, function to establish contacts with other cytoskeletal or membrane components to form a structure capable of resisting the retraction force generated by the actomyosin system. Microscopic observations in previous studies [33–36] have shown that MAPs interact with adjacent structures. The present study is the first to indicate (although indirectly) a similar type of interaction with a functional approach. The observation that transfection of MT actin-crosslinking factor resulted in inhibition of LPA-induced neurite retraction [37] is consistent with our hypothesis. In addition, our proposal is strongly supported by the resistance to retraction (induced by serum) conferred on CAD cell processes by the expression of GFP–Tau (Fig. 10). An alternative hypothesis, which is also based on the interaction of MTs with adjacent structures to confer resistance to retraction, is that arising from our previous findings regarding the interaction of MTs with the sodium pump (Na^+/K^+-ATPase) [38–40]. Because this ATPase is an integral membrane protein, we visualize such an interaction as an 'anchoring' of MTs to the membrane that is involved in the formation of a structural complex resistant to neurite retraction.

Experimental procedures

Chemicals

Nocodazole, paclitaxel (Taxol), TSA, 1-oleoyl-LPA, ADA, cytochalasin D, culture media, the Ac-Tub mouse mAb 6-11B-1, the Tyr-Tub mouse mAb Tub-1A2, the β-actin mouse mAb AC-15 and the total α-tubulin mouse mAb DM1A were from Sigma-Aldrich (St Louis, MO, USA). Rabbit polyclonal antibodies specific to Glu-Tub and Δ2-tubulin were prepared in our laboratory as described previously [41]. IRDye 800CW goat anti-(mouse IgG) and IRDye800CW goat anti-(rabbit IgG) were from Li-Cor Biosciences (Lincoln, NE, USA). Lipofectamine 2000 and Cy3 antibody were from Invitrogen (Grand Island, NY, USA). Fetal bovine serum was from Natocor (Córdoba, Argentina). Y-27632 and FluorSave were from Calbiochem (Billerica, MA, USA).

Cell culture

CAD cells were cultured in DMEM/F12 (50 : 50, v/v) supplemented with 10% (v/v) fetal bovine serum, 10 units·mL^{-1} penicillin, and 100 μg·mL^{-1} streptomycin, and maintained in a humidified atmosphere (air/CO_2, 19 : 1) at 37 °C. Differentiation of cells was induced by removal of the culture medium and its replacement with fetal bovine serum-free medium. The differentiation status of cells was assessed by microscopic examination.

GFP–tubulin and GFP–Tau transfection

CAD cells were grown on coverslips in 24-well plates, and transfected with Lipofectamine 2000 reagent and the plasmid pEGFP–Tub (Clontech, Mountain View, CA, USA), and PcDNA3-GFP–Tau23 (a gift from M. Rasenick and G. Morfini, University of Illinois at Chicago, IL, USA). The manufacturer's protocol was modified by the addition of fetal bovine serum (final concentration of 1%) and 1.3 μL of Lipofectamine 2000 per reaction. After 6 h, differentiation of cells was induced by removal of the culture medium and its replacement with fetal bovine serum-free medium. Cells were fixed with NaCl/P_i containing 4% paraformaldehyde and 3% sucrose for 10 min, and the coverslips were mounted with FluorSave.

Immunofluorescence

Cells were cultured on coverslips, fixed with NaCl/P_i containing 4% paraformaldehyde and 3% sucrose for 10 min, and then extracted with NaCl/P_i containing 0.25% Triton X-100. The samples were washed with NaCl/P_i, incubated with 5% (w/v) BSA in NaCl/P_i for 1 h, and then incubated with the primary antibody for 4 h at 37 °C.

After three washes with $NaCl/P_i$, cells were incubated for 1 h at 37 °C with Cy3 anti-mouse IgG at a 1 : 2000 dilution. Coverslips were mounted in FluorSave (Calbiochem, San Diego, CA, USA), and fluorescence was observed on a Confocal Spectral version FLOWVIEW 1000 (Olympus). When a comparison of different preparations was necessary, photographs were taken with the same gain value.

FRAP

Undifferentiated CAD cells were grown in a borosilicate chamber (Lab-Tek, Nalge Nunc, Waltham, MA, USA) and transfected with GFP–Tyr-α-tubulin. After 48 h under differentiating conditions, cells were subjected to FRAP analysis, i.e. photobleaching of GFP–tubulin in a defined area of the cell and measurement of fluorescence recovery in this area as a function of time. Fluorescence values in a background region and in a neighboring unbleached cell were also measured. Images of living cells in FRAP experiments were acquired with a 20 × 0.5 numerical aperture objective (Plan Neofluar; Carl Zeiss, Jena, Germany) with the confocal pinhole of the microscope fully open. Selective photobleaching of GFP was performed under a laser scanning confocal microscope (model FV1000; Olympus; Center Valley, PA, USA). Living cells were maintained at 37 °C in a 5% CO_2 atmosphere during image acquisition. Fluorescence quantification in selected regions of interest was performed with METAMORPH 4.5 (Molecular Devices, Sunnyvale, CA, USA).

Isolation of the cytoskeletal fraction

CAD cells were grown under differentiating conditions (fetal bovine serum-free medium) in capsules (diameter of 3.5 cm) for 5 days. To isolate the cytoskeletal fraction, a five-fold-concentrated MT-stabilizing buffer was added directly to each capsule to give final concentrations of 100 mM Mes (pH 6.7), 1 mM EGTA, 1 mM $MgCl_2$, 10% glycerol, 10 μM paclitaxel, and 5 μM phenylmethanesulfonyl fluoride. After 2 min, Triton X-100 was added (final concentration of 0.5%), and the cultures were maintained at 37 °C for 4 min with frequent gentle agitation. The residues were scraped off and transferred to plastic tubes, and the cytoskeletal fractions were separated from the cytosolic fractions by centrifugation (7000 ***g***, 5 min). The pellet fractions were washed with one-fold MT-stabilizing buffer, centrifuged at 20 000 ***g*** for 5 min, and resuspended in Laemmli sample buffer.

SDS/PAGE and western blotting

Proteins were separated by SDS/PAGE (10% gels) [42] and transferred to nitrocellulose sheets [43]. After blocking with 5% nonfat dried milk powder in $NaCl/P_i$, the sheets were incubated with various primary antibodies for 4 h at room temperature, in 1% nonfat dried milk powder in $NaCl/P_i$, as follows: anti-total α-tubulin IgG and anti-Ac-Tub IgG (1 : 5000 dilution); anti-Tyr IgG and anti-β-actin IgG (1 : 3000 dilution); anti-Glu serum and anti-Δ2-tubulin serum (1 : 500 dilution). The sheets were washed three times, incubated with infrared fluorescent secondary antibodies for 1 h at room temperature (1 : 25 000 dilution), washed again, and scanned with an Odyssey infrared scanner (Li-Cor). Optical density of bands were quantified with SCION IMAGE (Scion Corp.; http://scion-image.software.informer.com/).

Microscopy and neurite length measurement

Phase contrast images were collected with an inverted microscope (Axiovert 135; Carl Zeiss) equipped with an Olympus XM10 camera and CELLSENS digital imaging software (Olympus). The length of each process was quantified with IMAGE J (National Institutes of Health, Bethesda, MD, USA). For each sample, five different areas were analyzed, and the sum of the lengths of the measured processes was divided by the number of cells. Cells without processes were excluded from the analysis. In some experiments, as indicated, the analysis of neurite length was performed on a single group of cells that were sequentially photographed over time.

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Author contributions

María E. Chesta has the main contributor as she has performed most of the experiments. Agustín Carbajal has performed some of the last experiments focusing on the statistics and counting of neurites and participated in discussion of most of the experiments. Carlos A. Arce participated in most discusions and with his experience has oriented some parts of the work.

Gastón Bisig initiated the study of CAD cells and controlled all the performance of the experiments and orientation of this work.

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