Rapid neurite outgrowth in neurosecretory cells and neurons is sustained by the exocytosis of a cytoplasmic organelle, the enlargeosome

Gabriella Racchetti[1], Anna Lorusso[1], Carsten Schulte[1,*], Daniela Gavello[2], Valentina Carabelli[2], Rosalba D'Alessandro[1] and Jacopo Meldolesi[1,‡]

[1]Division of Neuroscience, Vita-Salute San Raffaele University and Scientific Institute San Raffaele, National Institute of Neuroscience-Italy, IIT Network, Research Unit of Molecular Neuroscience, via Olgettina 58, I-20132 Milan, Italy
[2]Department of Neuroscience, University of Turin, Corso Raffaello 30, I-10125 Turin, Italy

*Present address: IFOM–FIRC Institute of Molecular Oncology Via Adamello 16, Milan, Italy
‡Author for correspondence (meldolesi.jacopo@hsr.it)

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Summary

Neurite outgrowth is known as a slow (days) process occurring in nerve cells and neurons during neurotrophin treatment and upon transfer to culture, respectively. Using Y27632, a drug that induces activation of Rac1, a downstream step of the neurotrophin signaling cascade, we have identified a new form of outgrowth, which is rapid (<1 hour) and extensive (>500 μm^2 surface enlargement/single cell/first hour). However, this outgrowth takes place only in cells (PC12-27 and SH-SY5Y cells, and embryonic and neonatal neurons) rich in an exocytic organelle, the enlargeosome. Golgi vesicles, TGN vesicles and endosomes are not involved. The need for enlargeosomes for plasma-membrane expansion was confirmed by the appearance of their marker, Ahnak, at the cell surface and by the dependence of neurite outgrowth on VAMP4, the vSNARE of enlargeosome exocytosis. In enlargeosome-rich cells, VAMP4 downregulation also attenuated the slow outgrowth induced by nerve growth factor (NGF). Similar to NGF-induced neurite outgrowth in enlargeosome-lacking cells, the new, rapid, Y27632-induced process required microtubules. Other properties of neurite outgrowth in cells lacking enlargeosomes – such as dependence on VAMP7, on microfilaments, on gene transcription and on protein synthesis, and blockade of mitoses and accumulation of neuronal markers – were not evident. The enlargeosome-sustained process might be useful for the rapid neurite outgrowth at peculiar stages and/or conditions of nerve and neuronal cells. However, its properties and its physiological and pathological role remain to be investigated.

Key words: Rac1, Nerve-cell differentiation, Neuronal primary cultures, PC12 cells, SH-SY5Y cells, Vamp4

Introduction

The neurite outgrowth taking place in neurons (during in vivo differentiation and upon establishment in primary cultures) and in neurosecretory cells [during exposure to neurotrophins, peptides and adhesion proteins (Huang and Reichardt, 2003; Ravni et al., 2006)] is known to last between many hours to a few days. During the process, a dynamic reorganization of the cytoskeleton is accompanied by the expansion of the cell surface dependent on the exocytosis of cytoplasmic vesicles that are distinct from canonical neurosecretory vesicles (Martinez-Arca et al., 2001; Morris and Homann, 2001; Tsaneva-Atanasova et al., 2009; Prager-Khoutorsky and Spira, 2009). In previous studies, a population of exocytic vesicles possibly including secretory lysosomes, identified by their tetanus-toxin-insensitive vSNARE, VAMP7, had been reported to sustain outgrowth in neurons and rat pheochromocytoma PC12 cells (Martinez-Arca et al., 2001; Arantes and Andrews, 2006). Whether additional organelles, not yet identified, are also (or alternatively) involved (Tang, 2008; Bonanomi et al., 2008) remained to be established. Here we show that the enlargeosomes, exocytic organelles (Borgonovo et al., 2002; Cocucci et al., 2004; Cocucci et al., 2008) expressed by some nerve cells and embryonic and neonatal brain cortex neurons, sustain a new, very rapid (tens of minutes) form of neurite outgrowth induced by the activation of the small GTPase Rac1 (Nusser et al., 2002; Aoki et al., 2005; Takefuji et al., 2007). In nerve cells lacking enlargeosomes, a Rac1-induced response occurs but resembles that induced by nerve growth factor (NGF) inasmuch as it is less extensive and delayed.

Results and Discussion

Rapid neurite outgrowth in PC12-27 and SH-SY5Y cells

Rac1, a downstream step of the neurotrophin signaling cascade, is known to operate under the inhibitory control of the Rho-associated protein kinase ROCK (Yamaguchi et al., 2001; Takefuji et al., 2007). In previous studies (Borisoff et al., 2003; Takefuji et al., 2007), inhibition of ROCK by a drug, Y27632, had been shown to induce neuron-like differentiation of nerve cells; however, this only occurred at slow rate. To reinvestigate the process, we used 10, 25 or 50 μM Y27632 in three types of neurosecretory cells. Here we report the results obtained with the highest concentration. With the others, the results were slower and less extensive but fully consistent.

In both a rat pheochromocytoma PC12 clone defective of neurosecretion [PC12-27 (Borgonovo et al., 2002)] (Fig. 1B,E,F; DIC time-lapse recording in supplementary material Movie 1) and the human neuroblastoma SH-SY5Y line (Fig. 1C) the response was prompt and rapid. Sprouting, which was visible in ~70% of the cells 10-20 minutes after application of Y27632, developed within 1 hour into neurite outgrowths exhibiting typical growth cones at their tips, with diffuse tubulin continuous with neurites and actin concentrated at filopodia and lamellipodia (Fig. 1E,F). Initially, there were either three to five branched and short

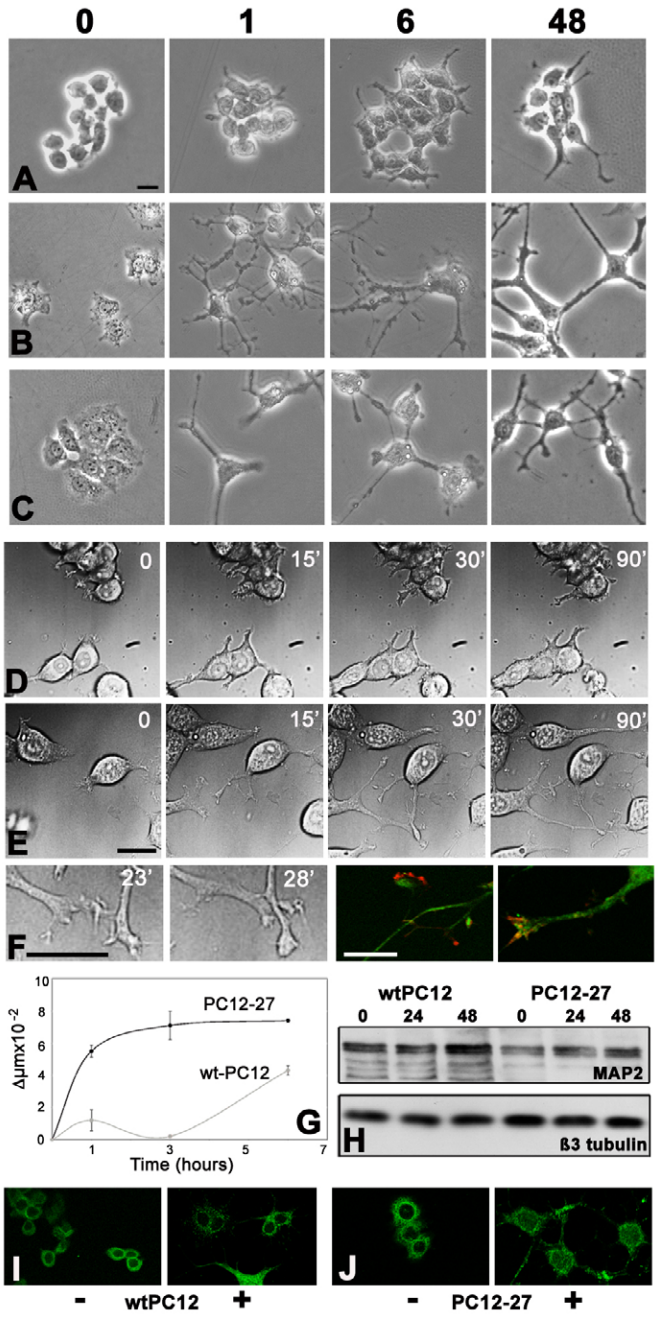

Fig. 1. Neurite outgrowth induced by Y27632 in wt-PC12, PC12-27 and SH- SY5Y cells. (**A-C**) Phase-contrast images of wt-PC12 (small and thick; A), and PC12-27 (B) and SH-SY5Y (C) (larger and flat) cells treated with Y27632 (50 μM) for the indicated times (hours). (**D**,**E**) Frames of the DIC time-lapse imaging (see supplementary material Movies 1, 2) are shown, illustrating the effects of Y27632 in wt-PC12 and PC12-27 cells. (**F**) Growth cones of outgrowing neurites of Y27632-treated PC12-27 cells are shown as phase contrast (from the DIC imaging supplementary material Movie 1; left) and dually immunolabeled (right; tubulin, green; actin, red) images. (**G**) The increases of cell surface area are shown, expressed as $\mu m^2 \pm$ s.e.m. (averages of 4-14 cells/time point), revealed by whole-cell patch-clamp capacitance assays in wt-PC12 and PC12-27 cells treated with Y27632. (**H**) Western blots of MAP2 and β3 tubulin in wt-PC12 and defective PC12 cells, before and after a 24- or 48-hour application of Y27632 are shown. (**I**,**J**) The β3-tubulin immunolabeling of wtPC12 (I) and PC12-27 (J) cells before (–) and after (+) Y27632 treatment (24 hours) is shown. Scale bars: 10 μm in A (valid for B, C, I, J), E (valid for D) and F.

(6.5±2.5 μm) neurites/cell, or one or two linear and longer (15.2±3.8 μm) neurites. As time progressed, long neurites became more frequent, and intermingled with those of adjacent cells to yield intricate networks (Fig. 1B,C). The corresponding surface expansion of the PC12-27 cells, revealed by patch-clamp capacitance assays, was on average >500 μm^2 at 1 hour, and ~700 μm^2 at 3 and 6 hours (Fig. 1G). By contrast, in wild-type PC12 cells (wt-PC12), neurite outgrowth became appreciable only after 6 hours (Fig. 1A,D,G; DIC time-lapse recording in supplementary material Movie 2). In non-nerve HeLa cells, the drug failed to induce any sprouting (supplementary material Fig. S1).

The Y27632-induced responses were limited to neurite outgrowth, without other aspects of NGF-induced differentiation (Greene et al., 1983; Huang and Reichardt, 2003). The levels and widespread distribution of neuronal markers (MAP2 and β3 tubulin) remained unchanged (Fig. 1H-J), whereas mitoses kept going not only in smooth but also in neurite-exhibiting PC12-27 cells (supplementary material Fig. S2A,B). Also at variance with NGF, the Y27632-induced neurite outgrowth took place even when gene transcription and translation were blocked by actinomycin D and cyclohexamide, respectively (supplementary material Fig. S2C). Dissociation of neurite outgrowth from these events had already been reported in another PC12 clone, PC12D (Sano et al., 1998). In that clone, however, the nature of the exocytic organelles sustaining neurite outgrowth had not been investigated.

Rapid neurite outgrowth is sustained by enlargeosome exocytosis

PC12-27 and SH-SY5Y cells express enlargeosomes (specific marker is Ahnak). By contrast, wt-PC12 lack these organelles (Borgonovo et al., 2002; Cocucci et al., 2008) (Fig. 2A and supplementary material Fig. S3). Therefore, enlargeosomes could sustain the rapid outgrowth responses. The level of Ahnak did not change during treatment with Y27632 (Fig. 2A); however, its intracellular distribution changed, from being spread throughout the whole cytoplasm to being concentrated at the cell periphery (supplementary material Fig. S3B,C). This move was due, at least in part, to enlargeosome exocytosis, documented by the increase of Ahnak surface immunolabeling at the body and neurites of PC12-27 and SH-SY5Y cells (Fig. 2B,C).

To investigate whether enlargeosome exocytosis was causally related to neurite outgrowth, experiments were performed with siRNAs specific for mRNAs encoding three VAMPs: VAMP4, a vSNARE of the Golgi and TGN area (Steegmaier et al., 1999) that is effective in only one regulated exocytosis, that of enlargeosomes (Cocucci et al., 2008); VAMP7, which has been shown to effect the exocytoses that sustain neurite outgrowth in wt-PC12 and neurons (Martinez-Arca et al., 2001); and VAMP8, which is operative only in intracellular membrane traffic (Antonin et al., 2000). In both PC12-27 and SH-SY5Y cells exhibiting high VAMP4 downregulation, both enlargeosome exocytosis and neurite outgrowth were greatly reduced or absent, whereas, in those downregulated for VAMP8, the processes seemed to be unaffected (Fig. 2D,E, middle and right images). In PC12-27 cells downregulated for VAMP7, both the rapid and the slow neurite outgrowth induced by Y27632 (1 hour) and NGF (50 ng/ml, 48 hours), respectively, and their surface Ahnak transfers were largely maintained (Fig. 2F).

The role of VAMP4 was investigated also in wtPC12 treated as above with Y27632 and NGF; results were compared with those from a subclone of PC12-27, PC12-27/trk108, transfected with the

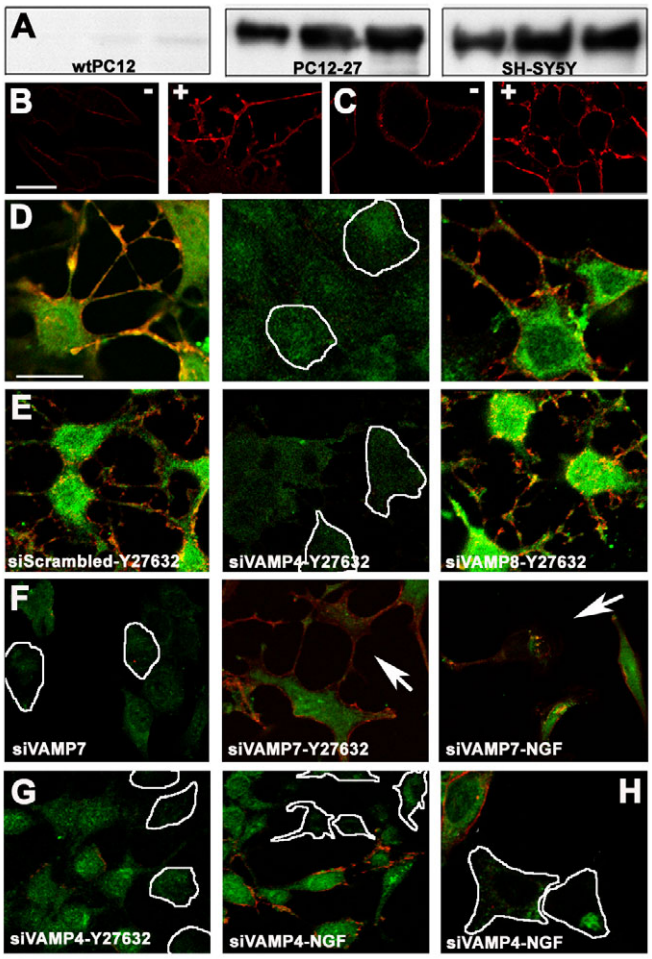

Fig. 2. Y27632-induced neurite outgrowth: dependence on enlargeosomes (VAMP4). (**A**) Western blots showing that levels of the enlargeosome marker Ahnak are almost inappreciable in wt-PC12 and high in PC12-27 and SH-SY5Y cells. These levels do not change significantly during treatment with Y27632 (50 μM) for 24 (middle) and 48 (right) hours. (**B**,**C**) Surface Ahnak (red), levels of which are very low in resting PC12-27 and SH-SY5Y cells (–), increases strongly during 1-hour incubation with Y27632 (+), documenting the extensive enlargeosome exocytosis occurring concomitantly with neurite outgrowth. (**D**,**E**) PC12-27 (D) and SH-SY5Y (E) cells were transfected twice (at 0 and 24 hours) with the indicated siRNAs and stimulated for 1 hour with Y27632 24 hours after the second transfection, then fixed, surface immunolabeled for Ahnak (red), permeabilized and immunolabeled for VAMP4 (green). In these and in following panels a few cells downregulated for a VAMP and exhibiting no surface Ahnak have been encircled in white. Notice that, at variance with the cells transfected with the siRNA, scrambled or specific for VAMP8 (strong green, left and right images), those downregulated for VAMP4 (faint green, middle images) exhibit no or short neurites and no surface Ahnak. (**F**) Shown are PC12-27/trk108 cells downregulated for VAMP7, then fixed when untreated (left) or exposed to Y27632 (1 hour, middle) or NGF (50 ng/ml, 48 hours, right) and immunolabeled for Ahnak (red) and VAMP7 (green). Notice that both the drug-induced and the neurotrophin-induced neurite outgrowth did take place in the VAMP7 downregulated PC12-27/trk108 cells (arrows). (**G**) Shown are wtPC12 cells downregulated for VAMP4, treated with Y27632 or NGF as in F and processed as in D and E. (**H**) Shown are PC12-27/trkA108 cells downregulated for VAMP4 and treated with NGF. (G,H) Notice that, in the downregulated wtPC12, Y27632 had no appreciable effect, whereas NGF kept its moderate outgrowth response, without enlargeosome exocytoses; in PC12-27/trkA108 cells, downregulation of VAMP4 prevented both NGF responses (compare to panel D, middle). Scale bar: 10 μm.

NGF receptor trkA. This cell clone was chosen because its non-downregulated cells exhibit outgrowth responses to the neurotrophin that are analogous to wtPC12 (Leoni et al., 1999). VAMP4-downregulated wtPC12 exhibited no change upon Y27632 treatment; their slow, moderate NGF-induced outgrowth response was apparently unaffected, whereas the discharge of the few enlargeosomes accumulated during differentiation (Cocucci et al., 2007) was blocked (Fig. 2G). By contrast, VAMP4 downregulation prevented both the slow outgrowth and the enlargeosome exocytosis induced by NGF in PC12-27/trk108 cells. Combined with those of Fig. 2F, these results confirm that, in PC12-27 cells, neurite outgrowth is mostly sustained by exocytosis of enlargeosomes whereas, in wt-PC12, the role of VAMP4 and enlargeosomes in outgrowth is minor, if any.

Enlargeosome exocytosis is known to depend on Ca^{2+} and PKC (Borgonovo et al., 2002; Cocucci et al., 2007). We wondered whether these messengers have a role also in Y27632-induced neurite outgrowth. However, resting $[Ca^{2+}]_i$ levels were unchanged by Y27632 (supplementary material Fig. S4A) and neurite outgrowth induced by the drug was unaffected by loading PC12-27 with BAPTA, a Ca^{2+} chelator (supplementary material Fig. S4A,B). Moreover, two PKC blockers, hispidin and rottlerin, completely inhibited the exocytosis induced by phorbol esters (supplementary material Fig. S4E) but failed to modify appreciably Y27632-induced neurite outgrowth (supplementary material Fig. S4C,D). The enlargeosome exocytosis that sustains outgrowth is therefore triggered by a signal so-far unknown messenger, distinct from Ca^{2+} and PKC.

Golgi- and TGN-derived vesicles and endosomes have no role in Y27632-induced neurite outgrowth

Other exocytic organelles known to participate in cell surface expansions during in vitro spreading and cytokinesis (Balasubramanian et al., 2007; Boucrot and Kirchhausen, 2007; Gauthier et al., 2009) could also be involved in the Y27632-induced outgrowth. However, brefeldin A, the drug that, together with Golgi and ER intermixing, induces blockade of Golgi– and TGN–plasma-membrane vesicle traffic, failed to block neurite outgrowth (supplementary material Fig. S5A). Likewise, no block was observed in PC12-27 cells that were either pre-treated with LY294002, an inhibitor of PI3 kinase, necessary for endosome recycling [supplementary material Fig. S5B (van Dam et al., 2002)], or transfected with dominant-negative constructs of ARF6, a small GTPase known to control the endosome traffic [supplementary material Fig. S5C (Prigent et al., 2003)].

Y27632-induced neurite outgrowth depends on Rac1

Neurite outgrowth induced by Y27632 depends on the activation of Rac1. PC12-27 cells transfected with this small GTPase exhibited a neuron-like phenotype analogous to that induced by Y27632 (Fig. 3A). Moreover, transfection of a dominant-negative Rac1 construct (Fig. 3B) and treatment with the anti-Rac1 peptide W56 and the Rac1 blocker NSC23766 (200 and 10 μM, 2 hours, not shown) prevented the Y27632-induced outgrowth response.

Many [but not all (Bosco et al., 2009)] effects of Rac1 are mediated by changes in the distribution and polymerization of actin microfilaments (Ridley, 2006), which could also be important for Y27632-induced neurite outgrowth. The process, however, was apparently unaffected by latrunculin A, a drug that induces sequestration of actin monomers with ensuing depolymerization of microfilaments, and jasplakinolide, an inhibitor of actin

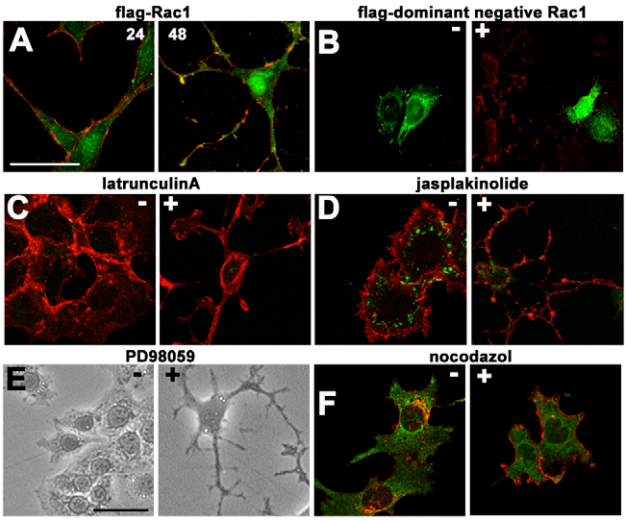

Fig. 3. Neurite outgrowth by Y27632 is induced via Rac1 activation and depends on microtubules. (**A**) Shown are PC12-27 cells co-transfected transiently with a constitutively active, flagged-Rac1 cDNA construct, and fixed 24 or 48 hours later. Notice the long neurites of transfected green cells, surface-positive for Ahnak (red), analogous to those of Y27632-treated cells (Figs 1, 2). (**B**) PC12-27 cells transfected with the dominant negative construct of Rac1 exhibited neither neurites nor surface Ahnak before (–) or after (+) treatment with Y27632 (1 hour, 50 μM). (**C-F**) Treatment with latrunculin A (5 μM, 15 minutes), jasplakinolide (100 nM, 2 hours) or the MAP-kinase inhibitor PD98059 (50 μM, 2 hours) had no appreciable effect on the neurite outgrowth induced by Y27632 (right images, C-E, +), which, by contrast, was blocked by nocodazol (30 μM, 1 hour, F). Scale bars: 10 μm in A (valid for B-D and F) and E.

depolymerization that induces microfilament assembly in compact arrays (Fig. 3C,D). Likewise, apparently normal responses were observed in PC12-27 cells pre-treated with PD98059 (Fig. 3E), a blocker of MAP kinase. By contrast, nocodazol, which induces depolymerization of microtubules, prevented Y27632-induced neurite outgrowth (Fig. 3F). Therefore, the change of cell architecture induced by Rac1 activation seems to depend on enlargeosome exocytosis combined with microtubule polymerization.

Neurite outgrowth sustained by enlargeosome exocytosis occurs also in neurons

Expression of the enlargeosome marker Ahnak in primary cultures of rat embryonic neurons had already been shown by immunocytochemistry (Borgonovo et al., 2002). In the adult rat brain tissue, however, western blots had revealed very low levels of the marker (Borgonovo et al., 2002). Fig. 4A confirms this result but shows the expression of Ahnak to be much higher in the brain of embryonic (E16) and postnatal (P2) rats compared with mature brain. Immunolabeling of brain cortex slices from P2 rats confirmed that many neurons positive for neurofilaments also exhibited variable levels of Ahnak, distributed in the cytoplasm (Fig. 4B). Additional Ahnak-positive cells in the slice are astrocytes positive also for GFAP (not shown).

To establish whether Y27632 induces enlargeosome exocytosis and neurite outgrowth also in neurons, we used primary cultures freshly (0-18 hours) dissociated from the P2 rat brain. At this stage, untreated neurons, positive for the specific markers MAP2 and β3 tubulin (Fig. 4C,D, upper frames), appear non-polarized, either smooth or exhibiting initial sprouting. Y27632 induced rapid and intense outgrowth of fibers, which were positive for MAP2 and β3 tubulin and decorated by Ahnak (Fig. 4C,D, lower frames). These fibers were much longer than the sprouts of untreated cells, ~tenfold after 1 hour and ~15-fold after 3 and 18 hours of Y27632 treatment (Fig. 4E).

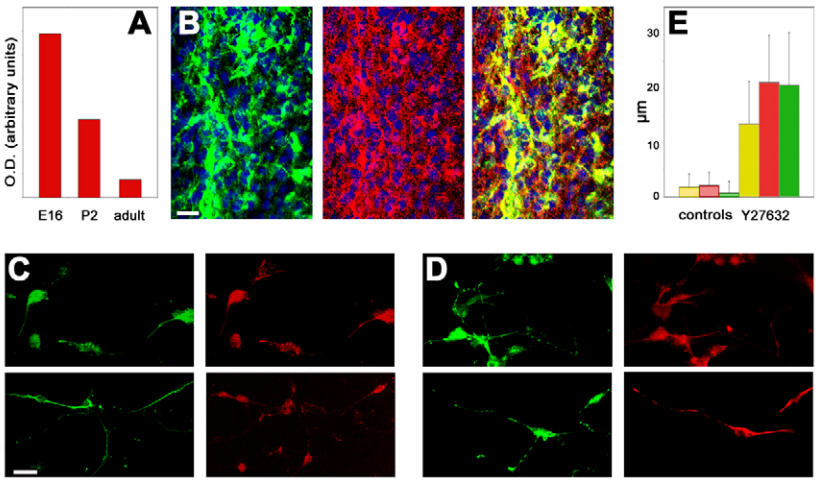

Fig. 4. Expression of Ahnak and neurite outgrowth in rat P2 neurons. (**A**) The levels of Ahnak, revealed by western blotting in the total brain of embryonic (E16), postnatal (P2) and adult rats, are shown by the histograms. (**B**) Shown is the expression of Ahnak (red) in cortical neurons of P2 rats dually immunolabeled for neurofilaments (green). The merged image is shown on the right. Nuclei are labeled by DAPI in all images. (**C**,**D**) Images of rat P2 neurons 18 hours after dissociation into primary culture, treated (lower row) or not (upper row) with Y27632 (50 μM, 1 hour), and then dually immunolabeled for MAP2 (green, C) or β3 tubulin (green, D) together with Ahnak (red). Notice the long Ahnak-positive neurites induced by the drug. The additional Ahnak-positive cells negative for MAP2 visible in C (upper row) are astrocytes. (**E**) Histograms of mean neurite lengths in neuronal-marker-positive cells, cultured for 18 hours with a change of medium without (controls) or with Y27632 during the last hour (yellow), the last 3 hours (red) or the whole 18 hours (green).

Conclusions

By using Y27632 to bypass neurotrophin receptors and the initial steps of their signaling cascades, we have identified in two neurosecretory cell lines (PC12-27 and SH-SY5Y), and also in primary cultures of freshly dissociated P2 rat neurons, a new, rapid process of neurite outgrowth, governed by Rac1 and sustained by the exocytosis of a specific organelle, the enlargeosome. This process, which is triggered, although slowly, in enlargeosome-rich cells also by NGF, takes place via the establishment of typical growth cones and requires microtubule polymerization. Other cells competent for neurite outgrowth, including wtPC12 and mature neurons, are poor or free of the organelle. Therefore, their neurite outgrowth seems to be sustained by other types of vesicles, such as those positive for VAMP7 (Martinez-Arca et al., 2001; Arantes and Andrews, 2006). At the moment, knowledge about the enlargeosome-dependent neurite outgrowth is still limited. In particular, nothing is known about the functional polarization and activity of its neurites. Considering, however, that various cell types, including embryonic and early neonatal neurons, express high levels of enlargeosomes, it seems reasonable to hypothesize a significant role in the physiology, and possibly also in the pathology, of these cells. Further studies are required to test this hypothesis.

Materials and Methods

Sources

wt-PC12 and PC12-27 clones, SH-SY5Y cells, anti-Ahnak monoclonal and anti-VAMP4 antibodies, and the siRNAs for VAMP4, VAMP7, VAMP8 and scrambled were as in Cocucci et al. (Cocucci et al., 2008); PC12-27/trk108 as in Leoni et al. (Leoni et al.,1999); HeLa cells as in Borgonovo et al. (Borgonovo et al., 2002); ionomycin, Fura-2 and BAPTA-AM as in Cocucci et al. (Cocucci et al., 2004). The flagged, constitutively active (G12V) and dominant-negative (T17N) chicken Rac1 and the HA-ARF6 constructs were gifts of Ivan DeCurtis (San Raffaele Institute, Milan, Italy) and Michel Lazdunski (Institut de Pharmacologie Moléculaire et Cellulaire, Sophia Antipolis, France). Murine anti-Rac1 was from BD Bioscience, San José, CA; anti-β3-tubulin from Covance, Berkeley, CA; anti-NeuN and anti-neurofilaments from Millipore, Billerica, MA; anti-GFAP from Dako, Glostrup, Denmark; Y27632 from Calbiochem, La Jolla, CA; NGF from Alomone, Jerusalem, Israel; W56 peptide from Tocris, Ellisville, MO; latrunculin A and jasplakinolide from Invitrogen, Eugene, OR; anti-MAP2, NSC23766, actinomycin, brefeldin A, cyclohexamide, nocodazol, hispidin, LY294002, PD98059 and rottlerin from Sigma-Aldrich Italy, Milan, Italy.

Cell processing, cultures and incubations

PC12 and SH-SY5Y cells were cultured and incubated as in Cocucci et al. (Cocucci et al., 2008); P2 rat brain tissue was either homogenized in 0.32 M sucrose using a Dounce homogenizer (western blotting), cut in small cubes (microscopy) or dissociated with papain (primary cultures) (Bonanomi et al., 2008).

Microscopy, immunofluorescence and $[Ca^{2+}]_i$ assays

Lines and primary cultures were fixed at –20°C for 4 minutes in Cytoskelfix (Cytoskeleton, Denver, CO) and washed. Immunolabeling of whole cell or surface only was carried out in cells permeabilized or not with 0.4% saponin before application of the first antibody (Cocucci et al., 2008). Small cubes of brain tissue were fixed in 4% formaldehyde for 2 hours, washed and embedded. Sections of 5 μm were doubly immunostained for Ahnak with neurofilaments or NeuN and stained with DAPI. $[Ca^{2+}]_i$ recordings were as in Cocucci et al. (Cocucci et al., 2007).

DIC time-lapse imaging

DIC time-lapse imaging was performed at the IFOM-IEO Campus, Milan. wt-PC12 and PC12-27 cells were plated at ~200,000 cells/ml on 35-mm glass-bottomed dishes (MatTek) coated with 100 μg/ml poly-D-lysine. After 24 hours in starvation medium they were incubated at 37°C, under 95% O_2-5% CO_2, on the stage of an inverted microscope (IX70, Olympus) equipped with a DIC prism. Recording (one image/9 seconds for 90 minutes; MetaMorph software of Universal Imaging) was started immediately after Y27632 addition.

Cell surface area

Cell surface area was estimated in wt-PC12 and PC12-27 cells patch-clamped at 0, 1, 3 and 6 hours after Y27632 addition. Electrophysiological recordings were performed with an EPC-9 patch-clamp amplifier using the PULSE software (HEKA Electronic, Lambrecht, Germany). Pipettes were fire-polished to obtain a final series resistance of 2-3 MΩ. In the whole-cell configuration, series resistance (between 5 and 10 mW) was monitored throughout the experiment. Intracellular solution contained (mM) 95 CsCl, 30 TEACl, 10 EGTA, 2 $MgCl_2$, 10 HEPES, 8 glucose, 2 ATP, 0.5 GTP, 15 phosphocreatine, 0.25 cAMP (pH 7.3). The bath contained (mM) 130 NaCl, 4 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 glucose (pH 7.4). Membrane capacitance measurements were corrected, as soon as the whole-cell configuration was reached, by canceling the slow capacitive currents charging the cell membrane. The pF values were then converted to $\mu F/cm^2$ as described (Cole, 1968).

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/2/165/DC1

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