PIP_3 is involved in neuronal polarization and axon formation.

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SUMMARY

Recent experiments in various cell types such as mammalian neutrophils and *Dictyostelium discoideum* amoebae point to a key role for the lipid product of PI 3-kinase, PIP_3, in determining internal polarity. In neurons, as a consequence of the elongation of one neurite, the axon is specified and the cell acquires its polarity. To test the hypothesis that PI 3-kinase and PIP_3 may play a role in neuronal polarity, and especially in axon specification, I observed the localization of PIP_3 visualized by Akt-PH-GFP in developing hippocampal neurons. I found that PIP_3 accumulates in the tip of the growing processes. This accumulation is inhibited by addition of PI 3-kinase inhibitors. Those inhibitors, consistently with a role of PIP_3 in process formation and elongation, delay the transition from stage 1 neurons to stage 3 neurons, and both axon formation and elongation. Moreover when the immature neurite contacts a bead coated with laminin, a substrate known to induce axon specification, PIP_3 accumulates in its growth cone followed by a rapid elongation of the neurite. In such conditions, the addition of PI 3-kinase inhibitors inhibits both PIP_3 accumulation and future axon elongation. These results suggest that PIP_3 is involved in axon specification, possibly by stimulating neurite outgrowth. In addition, when a second neurite contacted the beads, this neurite rapidly elongates whereas the elongation of the first laminin-contacting neurite stops, consistently with the hypothesis of a negative feedback mechanism from the growing future axon to the other neurites.

INTRODUCTION

Recent experiments showed the importance of the lipid products of PI 3-kinase in determining and maintaining internal polarity especially in neutrophil cells and *Dictyostelium* (Rickert et al., 2000; Chung et al., 2001; Wang et al., 2002). PI(3,4,5)P_3 (PIP_3) has a strong polarity during chemotaxis. The internal gradient of PIP_3 exceeds that of the external chemoattractant gradient and is one of the most upstream signaling molecules known to do so during polarization (Servant et al. 2000). A widely used probe for detecting the spatial distribution of the PI 3-kinase products is Akt-PH-GFP. Akt-PH-GFP, the pleckstrin homology domain of Akt tagged with green fluorescent protein interacting specifically with PI(3,4,5)P_3 and PI(3,4)P_2 (Blomberg et al., 1999; Gray et al., 1999), is recruited selectively to the membrane at the leading edge of the cell after exposure of fibroblasts, neutrophils or *Dictyostelium* to chemo-attractants. In addition, exogenous PIP_3 induces cell polarity in neutrophils not exposed to chemo-attractant, and inhibition of PI 3-kinase disrupts cell polarity. PI 3-kinase activity has been shown to function upstream of Rac and Cdc42 activation (Hawkins et al., 1995; Benard et al., 1999), but Rac and Cdc42 also function upstream of PIP_3 generation (Servant et al., 1999), indicating that PIP_3 and Rho GTPases probably function both upstream and downstream of one another in a positive feedback loop (Weiner et al., 2002).

Neurons polarize to elaborate multiple dendrites and one long axon, both of which differentiate from common immature neurites (Craig and Banker, 1994). These processes play

specific roles, the dendrites receive the information and the axon conveys it to other cells. Culture of rat hippocampal neurons is a useful system to observe the neuronal development, which was divided in 5 stages (Dotti et al., 1988; **Fig. 1)**. In stage 1, shortly after attachment to the substratum, neurons show small protrusions veils (lamellipodia) and later a few spikes (filopodia). These protrusions grow to be cylindrical extensions, and form a growth cone at the distal tip. Neurons then establish several short processes also called neurites (stage 2). At this stage, all neurites are roughly equal in length and alternate phases of elongation and retraction. Polarization occurs when one of the multiple neurites emerging from the cell body initiates a rapid phase of elongation (stage 3) whereas the others are still subjected to elongation/retraction phases. The specification of the axon is thought to depend on its length relative to the other minor processes (Bradke and Dotti, 2000). The remaining neurites begin to slowly elongate a few days later to become the dendrites (stage 4). Finally, neurons enter the phase of maturation in general 7 days after plating (stage 5). The molecular mechanisms underlying this polarization are still not completely cleared up.

A function of PI 3-kinase has been demonstrated recently in the survival of several neuronal types such as cerebellar granule neurons (D'Mello et al., 1997; Dudek et al., 1997; Miller et al., 1997) or sympathetic neurons (Crowder and Freeman, 1998). Several studies have implicated PI 3-kinase in neurite outgrowth in PC12 (Kimura et al., 1994; Kobayashi et al., 1997; Kita et al., 1998) and neuroblastoma cells (Sarner et al., 2000), possibly through Rac1 activation. PI 3-kinase

was also implicated in axon guidance (Ming et al., 1999). Recently, Shi et al. (2003) reported that selection of the future axon requires the activity of PI 3-kinase in association with a polarized distribution of mPar3 and mPar6. They showed the accumulation of mPar3 and mPar6 in the tip of the axon at stage 3 and found the PI-3 kinase activity to be localized there. These activities occur after the specification of the axon, however, and give us few indications about the events during the transition from stage 2 to 3.

In this study, using cultured hippocampal neurons, I determined when PI 3-kinase is activated and its phospholipid product PIP_3 accumulated, and whether this relocalization is important for neurite outgrowth and especially for the future axon growth in other words, whether PIP_3 is critical for axon specification. Taken together, my results suggest that PI 3-kinase and PIP_3 play an important role in axon formation.

MATERIALS AND METHODS

Antibodies and plasmids

Akt-PH-GFP was kindly given by Dr T. Balla (National Institutes of Health, Bethesda, Maryland, USA). I amplified and purified it by a Qiagen Endofree kit. Several studies have shown that some PH domains are specific for 3-phosphorylated inositol derivatives and, therefore, represent possible downstream targets of PI 3-kinase. Several PH domains, such as those of Akt, Btk and ARNO, have been shown to translocate to the plasma membrane after PI 3-kinase activation (For review, Blomberg et al., 1999).

Mouse Tau-1 antibody was from Chemicon (Pittsburgh, PA); polyclonal anti-SynapsinI was from Calbiochem (San Diego, CA); Texas-red or FITC conjugated secondary antibodies against mouse or rabbit IgG were from Amersham Bioscience, UK limited (Buckinghamshire, England).

Cell culture and transfection

Hippocampal neurons from rat E18 embryos prepared by use of papain as described previously (Berninger et al., 1993) were seeded on coverslips coated with poly-D-lysine (PDL) (Sigma, Saint Louis, MO) and laminin (LN) (Iwaki, Tokyo, Japan) or PDL only in Neurobasal Medium (Invitrogen, Carlsbad, CA) supplemented with B-27 supplement (Invitrogen) and 1 mM glutamine. Experiments were performed using neurons plated at low density (0.2 to 0.5 × 10^5 cells/well, 24-well plate) for morphological studies and at high density (2 × 10^5 cells/well, 24-well plate) for stripe and bead experiments. Before plating, neurons were transfected with the intended plasmids using the

calcium phosphate method as described previously (Inagaki et al., 2000). Briefly, dissociated neurons in the transfection buffer (Neurobasal Medium/B-27 supplement/1 mM glutamine/10 mM HEPES) with plasmid DNA were plated out on a 24-well hydrophobic plate and incubated for 90 min in 5% CO_2 at 37°C. The neurons were gently washed and detached from the well by pipetting. The collected cells were resuspended in the culture medium and plated out on the coated coverslips.

Preparation of PDL/LN pattern

Patterned PDL/LN on glass coverslips was prepared as described previously (Vielmetter et al., 1990) with slight modifications. Glass coverslips with diameters of 25 mm (Fisher Scientific, Pittsburgh, PA) or 13 mm (Matsunami, Osaka, Japan) were coated with PDL (1 mg/ml) overnight at 37°C. Coverslips were washed twice with phosphate-buffered saline (PBS) and then with H_2O. A silicon matrix (J. Jung, Tuebingen, Germany) with parallel channels of 50 μm width separated bars of 40 μm width was placed onto coverslips. A mixed solution containing 50 μg/ml LN and 0.1% bovine serum albumin (BSA) Texas Red conjugate (Molecular Probes, Eugene, OR) was used to fill the open channel through the inlet channel and incubated at 37°C for 2 h (Esch et al., 1999). Finally, the coverslips were washed and ready for seeding of hippocampal neurons.

LN-coated beads

LN-coated beads were prepared as follows: 4.5 or 10 μm diameter beads (Polysciences, Inc., Warrington, PA) were washed four times with PBS. Beads were then incubated in 50 μg/ml LN for 1 h at room temperature and washed with PBS.

Finally, beads were incubated for 30 min at 37°C with 1% heated BSA, washed, and used.

Immunofluorescence

Transfected neurons that had been cultured for 6 h were fixed with 3.7% formalin followed by treatment with ice-cold methanol for 10 min at -30°C, and blocked with 10% NGS. The cells were incubated with primary antibodies overnight at 4°C, washed, and incubated for 1 h with second antibodies. When stained with DiI or cell tracker Orange CMTMR (Molecular Probes), neurons were incubated with the marker prior to fixation. I considered that a cell showed accumulation if it was visible by microscope at 40x magnification. The percentage of accumulation refers to the number of cells exhibiting accumulation.

Analysis of neuronal morphology

Dissociated neurons grown at low density (with or without PI 3-kinase inhibitors) were used to determine morphological characteristics. Isolated neurons were randomly chosen from two or three coverslips and photographed with 40× magnification using an Axiophoto microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Cool Snap camera (Roper Scientific, Trenton, NJ). All processes and their branches were traced, their numbers were counted, and their lengths were measured using the LSM 510 software. A cell was considered to have an axon if the length of one process was at least twice as long as any other process and was more than twice the diameter of the cell body (Deitch and Banker, 1993) or if this process was Tau-1 positive. At least 20 cells for

each condition were examined in three separate experiments. When using PI 3-kinase inhibitors, they were replenished every 24 h if needed.

Time-lapse studies

Approximately 5-8 h after plating, the neuronal dishes were transferred to the incubator of an Olympus microscope (Olympus, Melville, NY). The beads were then added to the medium. Neurons were visualized by fluorescence. Of the transfected neurons, fluorescent images of each cell were collected every 1 min for about 1 h using a CCD camera (Roper Scientific) and Metamorph software (Universal Imaging Corporation, Downingtown, PA). The neurons that contacted beads during the recording session were then analyzed.

RESULTS

Akt-PH-GFP accumulates in developing hippocampal neurons

In the first set of experiments, localization of PIP_3 by indirect visualization using Akt-PH-GFP was checked in hippocampal neurons at different stages. Shi et al. previously mentioned that EGFP tagged PH domain from the serine threonine kinase Akt (PHAkt-EGFP) compromised the axon formation. In the system I used, to avoid any interference, expression of Akt-PH-GFP was very low and I observed the neurons not later than 16 h after transfection and generally from 6 to 12 h after. In these conditions, using a highly purified plasmid, Akt-PH-GFP transfected cells develop as the control cells.

In stage 1 neurons, Akt-PH-GFP accumulated in one part of the cell body, preferentially in the lamellipodial structures **(Fig. 2).** In stage 2 neurons, the accumulation was found especially in the tip of the growing neurites **(Fig. 2)** (number of neurites per neuron = 3.4, number of accumulation per neuron = 1.3, n=85). It is interesting to observe that although most of the neurons showed only one accumulation, some of them presented 2 or 3 accumulations **(Fig. 3A and 3B)** consistently with a possible competition between neurites (Dotti et al., 1988). Because control plasmid GFP-GST was diffuse in the neuron **(Fig. 2),** this accumulation was neither an artifact caused by an increased amount of membrane ruffling in the lamellipodia or in the growth cone nor it was caused by an increase of cytoplasmic volume. Furthermore, clear evidence was obtained by intensity imaging of Akt-PH-GFP and the cytoplasmic marker CMTMR **(Fig. 3A)** or the fluorescent membrane dye DiI **(Fig. 3B).** Although membranes (DiI fluorescence) and

cytoplasm (CMTMR fluorescence) could indeed be in higher abundance in the edge of lamellipodia or in the growth cone, the increase of Akt-PH-GFP was much greater.

In addition, EGFP-Tiam1-PH, also known to bind PIP_3 (Rameh et al., 1997) had the same behavior than Akt-PH-GFP (data not shown), whereas EGFP-PLCδ1-PH, which binds to $PI(4,5)P_2$ or IP3 (Lemmon et al., 1995, Varnai and Balla, 1998) did not accumulate (data not shown).

Akt-PH-GFP accumulation is PI 3-kinase dependent

To test whether PIP_3 accumulation, revealed by Akt-PH-GFP localization, depends on PI 3-kinase activity, I used PI 3-kinase inhibitors such as Wortmannin and LY294002. In both cases the addition of PI 3-kinase inhibitors inhibited spontaneous accumulation of Akt-PH-GFP in lamellipodia or tips of the growing neurites **(Fig. 2, Fig. 4A and 4B).** Whereas 6 h after plating about 52% of the neurons (all stages) showed spontaneous Akt-PH-GFP accumulation, only 26% of LY294002-treated neurons and 15.3% of Wortmannin-treated neurons displayed such accumulation. In living cells, PI 3-kinase inhibitors also decreased Akt-PH-GFP accumulation in response to contact with LN-coated bead (percentage of living cells showing accumulation: DMSO, 59.9 ± 6.4%, n = 25; Wortmannin, 13.5 ± 0.9%, $p < 0.0005$, n = 15; LY294002, 21.6 ± 3.4%, $p < 0.001$, n = 17; see **Fig. 9C**).

Regarding the stage of neurons, it is interesting to note that percentage of Akt-PH-GFP accumulation was especially high in stage 1 neurons and even higher in early stage 3 neurons, when one neurite is a little longer than the others **(Fig. 4B).** At stage 2, however, the neurites are in a transient stage,

alternating between elongation and retraction, and may not always necessitate the recruitment of a high level of PIP_3 **(Fig. 4B).**

PI 3-kinase is critical for neuronal development

When I studied the effects of PI 3-kinase inhibitors on neuronal morphology, I found that both Wortmannin **(Fig. 4C)** and LY294002 (data not shown) delayed axon formation. The most significant alteration, which was observed after treatment with PI 3-kinase inhibitors, involved a dramatic decrease in the number of cells displaying processes and hence entering stages 2 and 3 of neuronal development.

At day in vitro (DIV) 0, 6 h after plating, less than 30% of neurons were still at stage 1 in control experiment, whereas the percentage of stage 1 neurons increased to 40% in Wortmannin-treated cells **(Fig. 4C).** At DIV 1 the effect was even clearer, with less than 5% of stage 1 neurons in control culture and more than 45% in Wortmannin-treated cells **(Fig. 4C).** At DIV 2 or 3, in the presence of PI 3-kinase inhibitors, if neurons were at stage 3, both Wortmannin and LY294002 significantly inhibited axonal elongation and axonal branching **(Fig. 5A).** In such neurons, the polarity seemed clearly established, at least regarding axonal marker Tau-1 localization **(Fig. 5B).** However, not all neurons showed a polarized morphology **(Fig. 5B).** When I took a look at the neurite formation, I noticed that their number was not decreased by PI 3-kinase nor was the branching of neurites. Taken together, these results suggest that inhibition of PI 3-kinase activity revealed by the inhibition of Akt-PH-GFP accumulation delays axon specification but do not inhibit

neurite formation. On the other hand, I can't rule out the possibility that PI 3-kinase inhibition arrests the neuronal differentiation.

Contact with LN stripes rapidly induces PIP_3 accumulation

Esch et al. (1999) reported that contact with LN can govern which neurite can become the axon. To determine whether such a substrate can induce the translocation and accumulation of PIP_3, Akt-PH-GFP-transfected dissociated hippocampal neurons were grown on coverslips patterned with alternating stripes of LN and PDL. The pattern was confirmed by using anti-LN antibody (data not shown) or by visualization of Texas Red conjugated BSA mixed with LN solution.

When neurons developed for 24 h under these conditions, the axons almost always originated from processes that contacted LN (Esch et al., 1999; **Fig. 6**). I observed the behavior of transfected cells and Akt-PH-GFP localization in neurites reaching LN **(Fig. 6).** In more than 40% (n = 31) of stage 2 neurons I found that PIP_3 accumulation occurred in the process reaching LN.

To quantify the ability of LN to induce PIP_3 accumulation, I compared the percentage of processes contacting each substrate with the percentage of PIP_3 accumulation that occurred on each substrate **(Fig. 6).** The number of growth cones reaching each substrate was approximately the same (PDL, 47.1%; LN, 52.9%). However, 41.5% of PIP_3 accumulation occurred from the growth cones in contact with LN and only 9% from those in contact with PDL.

LN bead contact induces PIP_3 accumulation followed by a rapid

increase in the rate of neurite growth

In parallel with the stripe experiment used to direct axon specification, I also used a bead assay to observe the response of the neuron to LN in living sample. Addition of coated beads in the culture medium turned out to be a good method for local application of a specified molecule (Kuhn et al., 1995, 1998). In both cases, the process contacting LN displayed PIP_3 accumulation at the contact site **(Fig. 6A).** I speculated that both were equivalent in term of specification of axon and decided, for practical reasons, to use bead-coated method coupled with time-lapse to study the role of PIP_3 in axonal formation.

With this method, I investigated the changes that occur when a growth cone contacts LN, especially the Akt-PH-GFP localization. I selected transfected cells showing a diffuse localization of Akt-PH-GFP prior to contacting the bead. After contact with LN-coated beads, Akt-PH-GFP was translocated to the contact site **(Fig. 6, Fig. 7A-B, Fig. 8)**, it accumulated there and only then the neurite entered a rapid phase of elongation. Using time-lapse, I observed that translocation of Akt-PH-GFP occurred about 5 to 20 min after bead-neurite contact **(Fig. 7A-B, Fig. 8).** Akt-PH-GFP accumulated at the contact site of LN-coated bead (59.9 ± 6.4%, n = 25), whereas contact with BSA-coated beads did not induce such accumulation (14.6 ± 1.3%, n = 17). Although Akt-PH-GFP did not disappear from the cell body and other neurites to accumulate only at the LN contact site, there was a real translocation of Akt-PH-GFP, which seemed to be transported towards the contact site also revealed that PIP_3 accumulation was transient **(Fig. 7, Fig. 8).** I estimated the rate of elongation for 8 neurons. For the

neurite contacting the LN-coating bead, the rate of growth dramatically increased from 1.51 μm/h to 50.1 μm/h after PIP_3 accumulation. However the growth rate of the remaining neurites went from 2.2 μm/h to 0.15 μm/h **(also see Fig. 9A).** The addition of the PI 3-kinase inhibitors Wortmannin and LY294002 inhibited LN-induced Akt-PH-GFP accumulation (13.5 ± 0.9%, $p < 0.0005$, $n = 15$ and 21.6 ± 3.4%, $p < 0.001$, $n = 17$, respectively), and the neurite contacting LN did not show any modification in its growth rate **(Fig. 7C, Fig. 9B).**

DISCUSSION

Importance of PIP_3 and PI 3-kinase

Because the key events underlying axon specification can occur in a few minutes or so, I tried to use a convenient real-time monitoring method, which would allow us to study the importance of PI 3-kinase at early stages, including the critical period of transition between stages 2 and 3 when the axon is specified.

In this study, I showed that in hippocampal neurons, inhibition of PI 3-kinase activity and PIP_3 production and accumulation, which is monitored by Akt-PH-GFP, affected the development of neuronal polarity by inhibition of axon specification or elongation; the axon-like neurite when existed is clearly shorter and has less branching compared to non-treated neurons. However the establishment of the polarity is not completely inhibited. There are several explanations for these results. First, the drugs could be unstable over time. To overcome this, I changed the medium every 24 h and added fresh drugs, although degradation could occur within 24 h, allowing a slow polarization. Second, to avoid high mortality of neurons after exposure to the drugs, I used the lowest concentration reported to completely inhibit PI 3-kinase; a residual activity may be maintained in such conditions. Finally, it is quite possible that neurons possess an alternative pathway that could take place if the PI 3-kinase pathway fails.

By monitoring living Akt-PH-GFP transfected neurons using time-lapse experiment, I observed the behavior of PIP_3 and its relation to process elongation. When a LN-coated bead contacts

one neurite, this induces PIP_3 accumulation and a rapid elongation of the process follows this accumulation. Therefore PIP_3 may in fact act by enhancing the growth of one neurite, which would result in axon formation and polarization of the neuron. This hypothesis is consistent with previous observations that in neurons, as a consequence of the elongation of one neurite, the cell acquires its polarity (Bradke and Dotti, 2000).

One could ask why the percentage of neurons showing Akt-PH-GFP accumulation is not higher if PIP_3 is so critical. One possible reason is that this accumulation is in fact transient, as I saw during the time-lapse experiment. Another explanation is the limitation of the system. It is likely that PIP_3 level is critical, but the difference of level between neurites may be more critical than a strong accumulation in one if PIP_3 is implicated in the feedback loop. A small increase in PIP_3 in a lamellipodia or a growth cone could compromise the balance between a positive feedback signal and negative feedback signal without being detected by immunofluorescence.

Possible feedback regulation

A common scenario in establishing polarity and breaking the symmetry is the involvement of a positive feedback in all stage 2 growth cones; each neurite has the potential to grow further and become an axon. Therefore, to assure the growth of a single axon, a negative feedback is thought to be generated and propagated globally to slow the growth of the other remaining processes. Before stage 3, the positive and negative feedback loops are assumed to form an intricate balance. If the balance becomes unstable, the symmetry is broken and one

process—the future axon—enters a phase of rapid elongation and the growth of the other processes is inhibited (Andersen and Bi, 2000). When I used coverslips uniformly coated with LN to check the localization of the studied proteins, some neurons did not show only one but two or three accumulation points of Akt-PH-GFP. This probably indicates a competition between processes, which alternate between elongation and retraction just before axon specification (Dotti et al., 1988). However, by using striped coated material or coated beads I observed that neurons showing Akt-PH-GFP accumulation at the site of contact of LN almost never displayed the accumulation at another site. These results suggest that the specification could occur at the time of contact with LN and that PIP_3 could be a positive feedback factor leading to neurite elongation. These observations are consistent with the hypothesis that before specification several, if not all, neurites have the potential to become axon and that, even after the start of the polarization, nothing is completely fixed. Indeed experiments have shown that the formation of one axon is not an irreversible event but can be reversed by axotomy (Dotti and Banker, 1987; Goslin and Banker, 1989).

Characterization of neuron responses to LN bead contact

My video recordings indicated that neurons react with slight differences depending on the localization and the timing of the LN bead contact. I classified the observation into four categories **(Fig. 10)**. The first category concerns a neuron with several processes with only one of their growth cones contacting a LN bead. This contact leads to the accumulation of Akt-PH-GFP at the contact site and PIP_3

accumulation is followed by a rapid elongation of this process **(Fig. 7A, Fig. 9A).** The second category resulted from my observation that LN contact can drive a small filopodia to become neurite **(Fig. 7B).** Neurons of the third category initially have the same response as those in the first category but after a while one of the other neurites contacted a LN bead. This second contact leads to the cessation of elongation of the first LN-contacting neurite, the accumulation of Akt-PH-GFP at the new contact site, and rapid elongation of this second process. This observation is consistent with the hypothesis of a negative feedback created by the elongating neurite to decrease the rate of elongation of the other processes. However, sometimes it appears that two neurites contacting LN beads at the same time can elongate simultaneously (fourth category), possibly because positive feedback is too strong (eventually involving PIP_3) and cannot be overcome by the negative feedback.

Mechanism of axon specification

Rho GTPases family, molecular switches cycling between an active GTP-bound state and an inactive GDP-bound state (Nobes and Hall, 1994), play crucial roles in yeast budding and polarity establishment (Adams et al., 1990; Chant, 1994; Miller and Johnson, 1994). Neutrophil polarity was shown to be regulated by PIP_3- and Rho GTPase-mediated positive feedback (Wang et al., 2002). In neurons, axon formation is preceded by the appearance in one of the multiple neurites of a large growth cone containing a very labile actin network (Bradke and Dotti, 1997, 1999; Paglini et al., 1998). Given the importance of local actin dynamics in axon, the Rho GTPases are likely to

be involved in neuronal polarity. Indeed Rho GTPases are involved in the growth and retraction of axons and neurites (Nishiki et al., 1990; Jalink et al., 1994; Luo et al., 1994, 1996), with Rho acting as a negative regulator of neurite/axonal growth and Rac and Cdc42 acting as positive regulators **(Fig. 11).** Inactivation of all members of the Rho family GTPases in stage 2 hippocampal neurons with *Clostridium difficile* toxin B (Aktories, 1997) induces complete actin loss and prevents the establishment of neuronal polarity (Bradke and Dotti, 1999). The Rho family GTPases are controlled by three classes of regulators: GEF (guanine nucleotide exchange factors), GAP (GTPase-activating protein) and GDI (guanine nucleotide dissociation inhibitor protein). Among the GEFs expressed in the nervous system, Tiam1 (T-cell lymphoma invasion and metastasis protein) and STEF (SIF and Tiam1 like exchange factor), two guanosine nucleotide exchange factors for Rac1, are good candidates as upstream signaling molecules that likely regulate activities of Rho GTPases during the establishment of neuronal polarity. Tiam1 and STEF are shown to regulate neurite outgrowth in N1E-115 cells via Rac activation (Leeuwen et al., 1997; Matsuo et al., 2002, 2003).

In hippocampal neurons, overexpression of Tiam1 induces several axon-like neurites, whereas suppression of Tiam1 prevents axon formation (Kunda et al., 2001). My present results are consistent with these observations: both PIP_3 and Rac1 (data not shown) accumulate in the tip of the future axon and both have their localization disrupted by inhibitors of PI 3-kinase, which inhibited the polarization of the neuron.

Interestingly several effectors of Rho family members seem to affect the neuronal polarization. Wiskott-Aldrich

syndrome family protein (WASP and neural WASP, N-WASP), one of the Cdc42 effectors, is essential for Cdc42-induced filopodia formation (Miki et al., 1998a). Overexpression of N-WASP mutant, which disrupts the association with Cdc42, abolishes neurite extension both in neuroblastoma and in cultured hippocampal neurons (Miki et al., 1998a; Banzai et al., 2000). Proteins of the WAVE/SCAR group (WAVE 1-3), which are also members of the WASP family and function as downstream effectors of Rac (Miki et al., 1998b), are enriched in the growing CNS axon and play an essential role in axon development of *Drosophila* (Zallen et al., 2002). Therefore, it is likely that in cultured hippocampal neurons PIP_3 is responsible for the polarization and induction of axon via Rac1 and potentially WAVE or N-WASP and their role on actin filaments **(Fig. 11).**

The Rho effector Rho-kinase also called ROK or ROCK mediates neurite retraction driven by activated Rho in N1E-115 and PC12 cells (Amano et al., 1998; Hirose et al., 1998; Katoh et al., 1998). Rho-kinase phosphorylates the myosin-binding subunit of myosin phosphatase (MBS), thereby inhibiting the dephosphorylation activity of myosin phosphatase toward the myosin light chain (MLC) of myosin II (Kimura et al., 1996; Fukata et al., 2001). In addition, activation of Rho-kinase negatively regulates the axon formation in cerebellar granules cells (Bito et al., 2000). These observations suggest that inhibition of the Rho/Rho-kinase pathway might be important for initial axon formation.

One substrate of Rho-kinase in the brain is CRMP-2, member of the CRMP/TOAD/Ulip/DRP family. Phosphorylation of CRMP-2 by Rho-kinase plays a role in growth collapse by LPA in

DRG neurons (Arimura et al., 2000). We previously showed that CRMP-2 plays a critical role in axon specification (Inagaki et al., 2001). CRMP-2 is enriched in the growing axon of cultured hippocampal neurons at stage 3 and its overexpression induces the formation of multiple axons whereas deleted mutants suppress axon formation. We also found that CRMP-2 acts by promoting microtubule assembly (Fukata et al., 2002). So I wonder about the possible relationship between PI 3-kinase activity and CRMP-2, as both seem to play a critical role in axon formation. It will be very interesting to see if there is any interaction between proteins now known to accumulate in the growing axon like CRMP-2, mPar3 and mPar6. Additional studies will be required to fully understand the mechanisms of axonal specification.

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FIGURE LEGENDS

Fig. 1 Establishment of neuronal polarity in cultured hippocampal neurons. Embryonic hippocampal neurons in culture acquire their characteristic polarized morphology in a sequence of well-defined stages (from Dotti et al., 1988). Stage 1, immediately after plating: lamellipodia (indicated by an arrow) surround the cell. Stage 2, within half a day: several short immature neurites (around 20 μm) emerge that are not distinguished either as the axon or as dendrites. Stage 3, within another 12 to 24 hours: polarity first becomes evident, as one of the immature neurites shows enhanced elongation and acquires axonal characteristics. Stage 4, after 2 to 3 days in culture: the remaining neurites acquire dendritic morphology. Stage 5: maturation of both axonal and dendritic arbors. Open arrowheads indicate the immature neurites. Black arrowheads indicate growth cones.

Fig. 2 Localization of Akt-PH-GFP during hippocampal neuron development. a-c, Micrographs showing the diffuse GFP-GST localization in stage 1 (a) or 2 (b, c) hippocampal neurons. d-f, Micrographs showing Akt-PH-GFP localization in stage 1 (d) or 2 (e, f) neurons. Note that Akt-PH fluorescence is localized at the tip of the neurite (arrow). g-i, Micrographs showing Akt-PH-GFP localization when neurons are treated with Wortmannin. Note that the Wotmannin-treated neurons (g-i) were selected to show the absence of AKT-PH-GFP accumulation although they present a phenotype similar to control cells. This does not represent the morphological change induced by Wortmannin, which is shown in Fig. 4C.

Fig. 3 Specificity of the Akt-PH-GFP accumulation. A, CMTMR observation. a-c, e-g, Fluorescence micrographs of Akt-PH-GFP (a, e) and CMTMR (b, f), with an overlay of the two images (c, g; CMTMR red and Akt-PH-GFP green) in hippocampal neurons at stage 1 (a-d) or 2 (e-i). Graphs d, h, i plot the intensity of Akt-PH-GFP fluorescence (green line) and CMTMR fluorescence (red line) above a line (h, i; white, long arrows).
B, DiI observation. a-c, e-g, Fluorescence micrographs of Akt-PH-GFP (a, e) and DiI (b, f), with an overlay of the two images (c, g; CMTMR red and Akt-PH-GFP green) in hippocampal neurons at stage 1 (a-d) or 2 (e-i). Graphs d, h, i plot the intensity of Akt-PH-GFP fluorescence (green line) and DiI fluorescence (red line) above a line (h, i; white, long arrows). Note that Akt-PH-GFP fluorescence is mainly in lamellipodia or in growth cone.

Fig. 4 PI 3-kinase inhibitors delay the neuronal polarization in parallel with the inhibition of Akt-PH-GFP accumulation. A-B, Graphs showing the percentage of cells displaying Akt-PH-GFP accumulation in the absence or presence of drugs in hippocampal neurons, which were treated with drugs at 3 h after plating and examined at 6 h (A) or 24 h (A, B). C, Graph showing the stage distribution of the neuronal population 6 and 24 h after plating. Note that Akt-PH-GFP accumulation is inhibited by LY294002 (10 μM, $p < 0.005$) and Wortmannin (200 nM, $p < 0.001$) (A). When comparing based on neuronal stage, PI 3-kinase inhibitors clearly inhibit Akt-PH-GFP accumulation at stage 1 and 3 (C) and accordingly delay the progression to stage 3 (B). For this experiment, I considered neurons that displayed the longest process to be at stage 3, even though

this process is not necessary Tau-1 positive.

Fig. 5 Effect of PI 3-kinase inhibitors on neuronal morphology at DIV 2. A, Bar graphs showing that treatment with PI 3-kinase inhibitors inhibit axon outgrowth ($p < 0.05$) and axon branching ($p < 0.005$). More than 80 neurons were observed per experiment for each treatment, with three separate experiments performed. B, Micrographs showing neuron at DIV 2. The cells were cultured in the absence of any drugs (a, b) or in the presence of PI3-kinase inhibitors Wortmannin (200 nM) (c, d) or LY294002 (10 μM) (e-h) and were fixed and doubled-labeled with a monoclonal antibody against Tau-1 (red) and a polyclonal antibody against Synapsin I (green). Note that Tau-1 and Synapsin I immunolabels localize in the distal part of the axon of stage 3 neurons, whether they are treated with PI 3-kinase inhibitors or not. In these cells, however, the axon, when present, is shorter and has less branching than in control cells. Arrows indicate the axon. Scale bar, 25 μm.

Fig. 6 Relocalization of Akt-PH-GFP induced by LN. A, Micrograph of non-transfected (a) or Akt-PH-GFP transfected neurons (b-d). b, Hippocampal neurons cultured on substrates patterned with alternating stripes of PDL and LN. Cells were fixed about 15 h after plating. c, d, Micrograph of living Akt-PH-GFP transfected hippocampal neurons cultured about 5 h before addition of beads. The pictures were taken 20 min after addition of the LN-coated bead. Note that contact with an LN stripe or LN-coated beads leads to Akt-PH-GFP accumulation at the contact site. B, Graphs showing the distribution of growth cone (GC) depending on each substrate. Note that the

distribution of the growth cone is almost equal on PDL and LN but that the axon is mostly on LN. Similarly, Akt-PH-GFP accumulation mainly occurs when the processes reach LN. Arrows point to Akt-PH-GFP accumulation. Scale bar, 25 μm.

Fig. 7 Effect of Akt-PH-GFP relocalization in response to a LN-coated bead. A-C, Time-lapse images showing localization of Akt-PH-GFP after contact with LN, in the absence (A, B) or in the presence (C) of PI 3-kinase inhibitor Wortmannin (200 nM). All the process growth cones were on PDL at the beginning of the recording. In control cells (A, B), immediately after the growth cone of one process contacts a LN-coated bead, Akt-PH-GFP translocates and starts accumulating at the site of contact and the process enters a phase of rapid elongation. Wortmannin (C) inhibits both accumulation of Akt-PH-GFP and the elongation of the contacting process. Arrow indicates the contact of the neurite to LN-coated bead. Arrowhead points to Akt-PH-GFP accumulation. Scale bar, 25 μm.

Fig. 8 High-power images taken from the region in Fig. 5A (at left) showing localization of Akt-PH-GFP before and after contact with LN-coated beads. Pictures were taken each minute for 64 min. Arrow indicates the contact of the neurite to LN-coated bead. Arrowhead points to Akt-PH-GFP accumulation. Scale bar, 5 μm.

Fig. 9 A rapid phase of elongation follows Akt-PH-GFP accumulation. A, B, Length of processes over time in response to contact with LN-coated beads, in Akt-PH-GFP transfected hippocampal neurons, in the absence (A) or presence (B) of

Wortmannin (200 nM). C, Percentage of living hippocampal neurons displaying Akt-PH-GFP accumulation in the absence or presence of PI 3-kinase inhibitors. Note that addition of PI 3-kinase inhibitors dramatically inhibits the translocation of Akt-PH-GFP (C) and in parallel inhibits the increase in the rate of neurite growth in response to LN contact (B compared to A)、$p<0.001$.

Fig. 10 Schematic drawings of morphology of hippocampal neurons in response to LN-coated bead contact.
(1) Soon after the LN-coated bead contacts a small filopodia, Akt-PH-GFP accumulates at the contact site. Then, the filopodia elongates to become process. (2) The LN-coated bead contacts a neurite and induces Akt-PH-GFP accumulation. This neurite then enters a rapid phase of elongation. (3) The LN-coated bead contacts one neurite and induces Akt-PH-GFP accumulation and neurite elongation as in (2). Then, a second bead contacts another neurite. The first neurite stops its elongation and the second one elongates. (4) Two beads contact neurites at the same time. Akt-PH-GFP accumulation occurs in both neurites, which then elongate.

Fig. 11 Prospective signaling through Rho family GTPases and CRMP-2 within the establishment of neuronal polarity.
Extracellular signals, such as extracellular matrix, growth factors, and guidance cues, activate the upstream regulators of Rho family GTPases through receptors. In the growing axon, Rac/Cdc42 are activated, and signals are enhanced by a positive feedback loop mediated by PI 3-kinase, PIP_3, and GEF. Downstream of Rac/Cdc42, effectors such as IQGAP and WASP/WAVE

regulate actin filaments and microtubules. Although CRMP-2 is not known to work downstream of Rac/Cdc42, it can be speculated that CRMP-2 plays a role in the signaling pathway regulating neuronal polarity involving PI 3-kinase and Tiam1. Note that some of the interrelationships have been established in non-neuronal cell lines and need confirmation in neurons.

Fig. 1

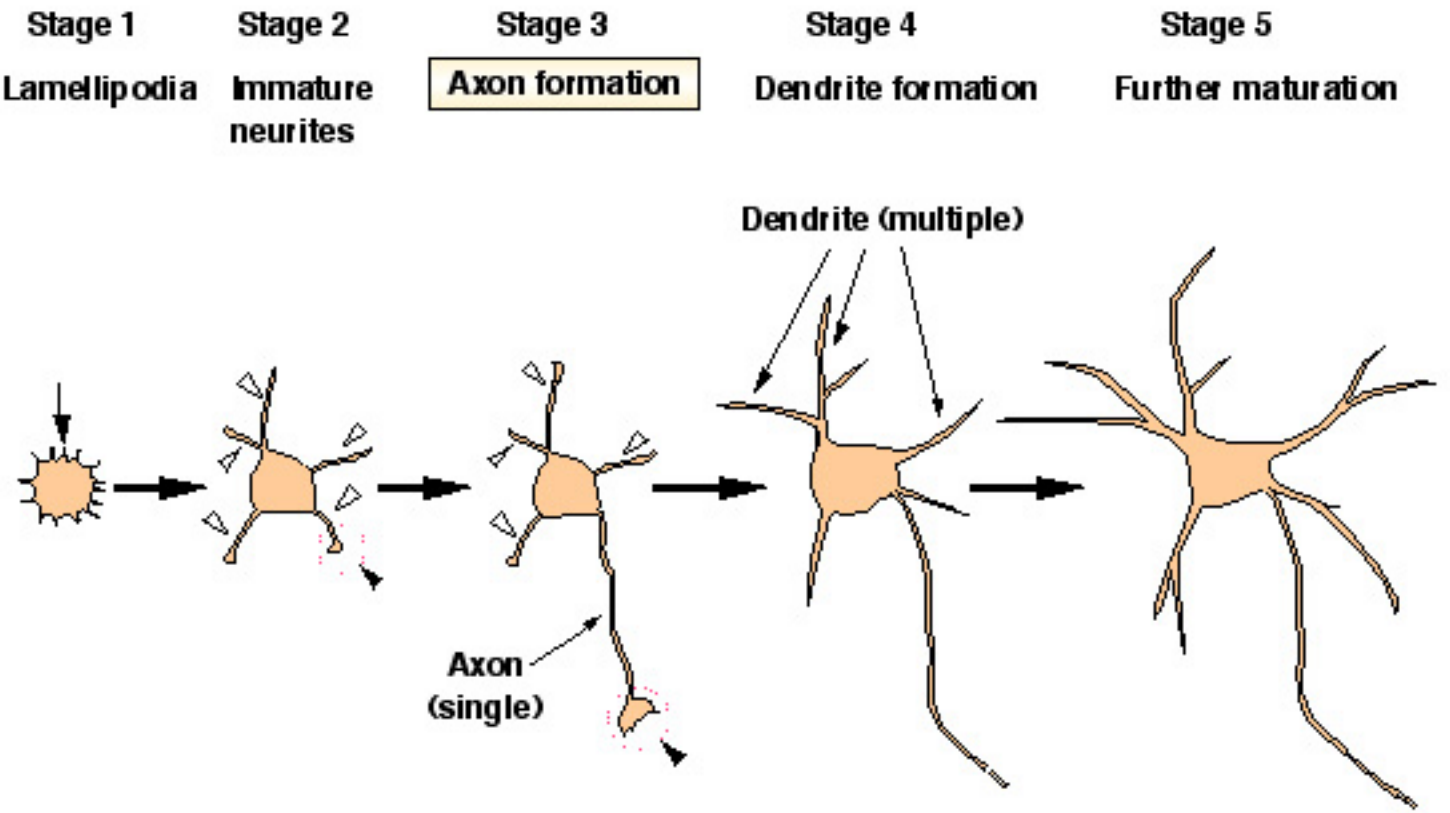

Stage 1
Lamellipodia
Stage 2
Immature neurites
Stage 3
Axon formation
Stage 4
Dendrite formation
Stage 5
Further maturation
Dendrite (multiple)
Axon (single)

Fig. 2

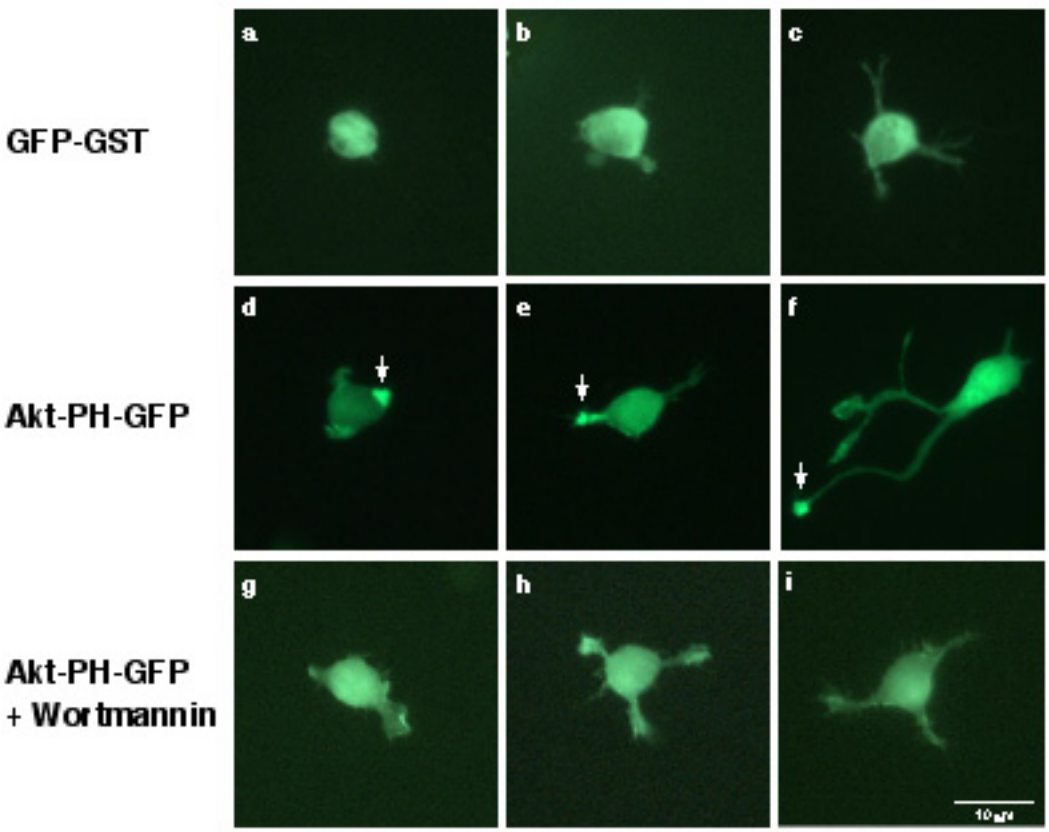


Fig. 3 A

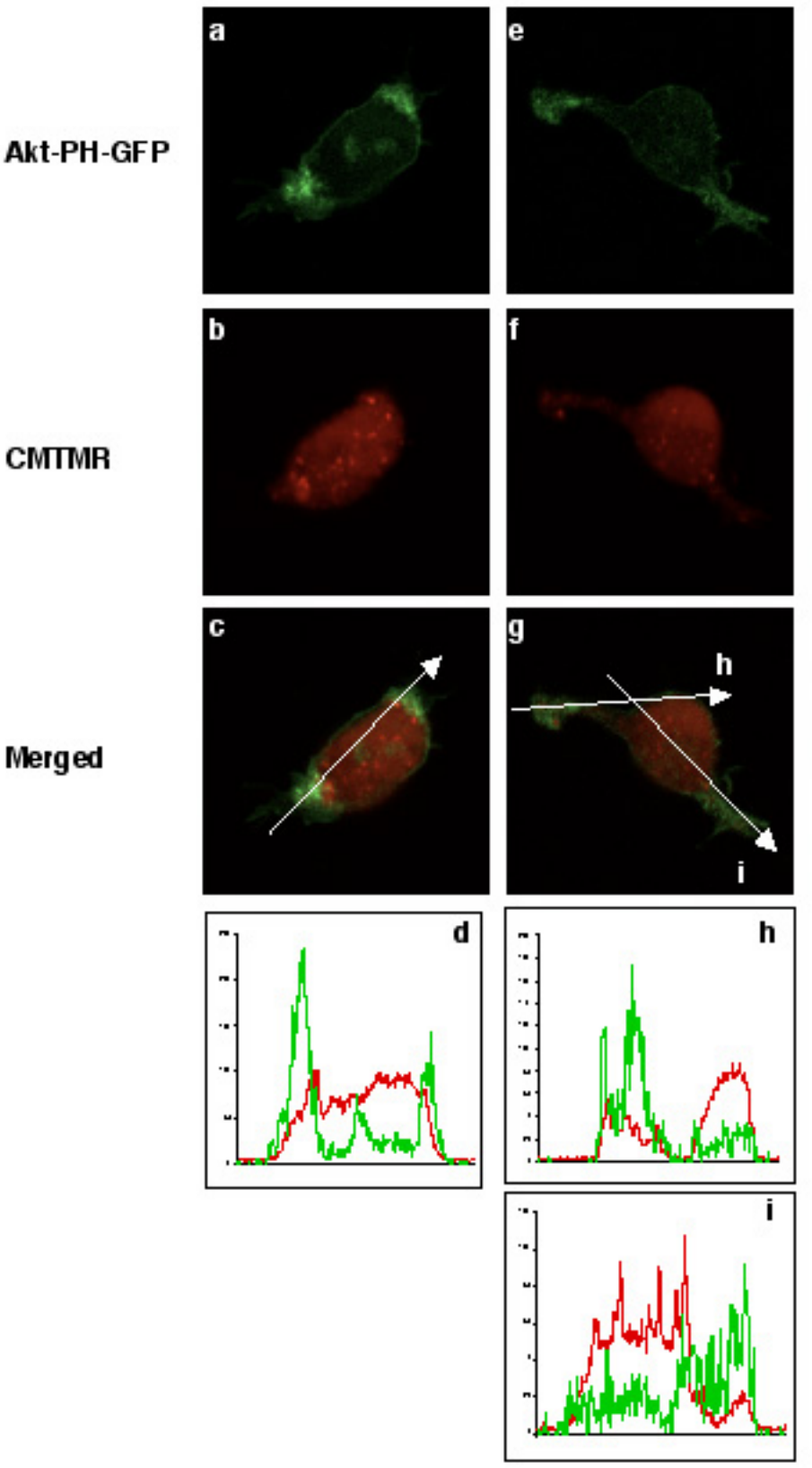


Fig. 3 B

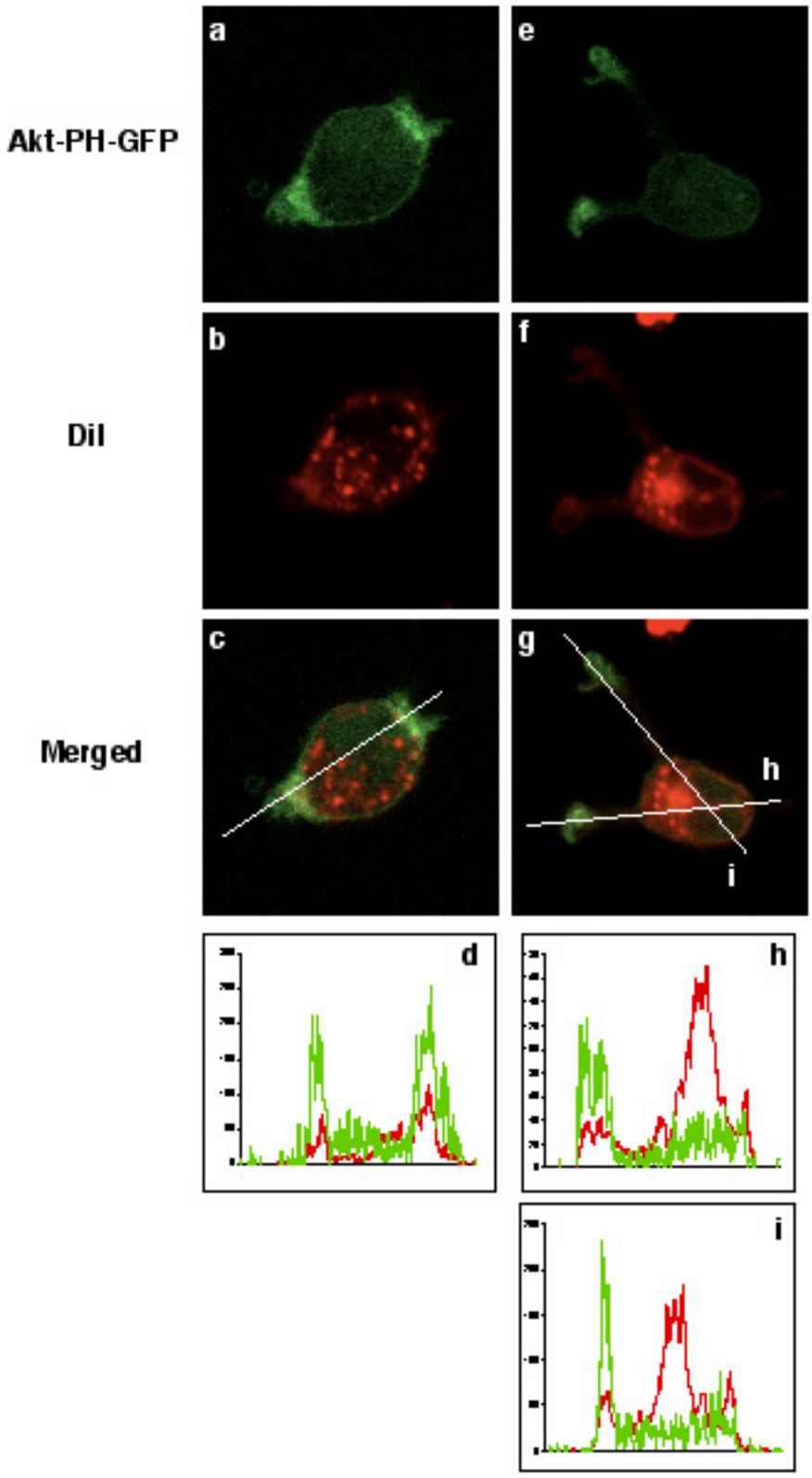
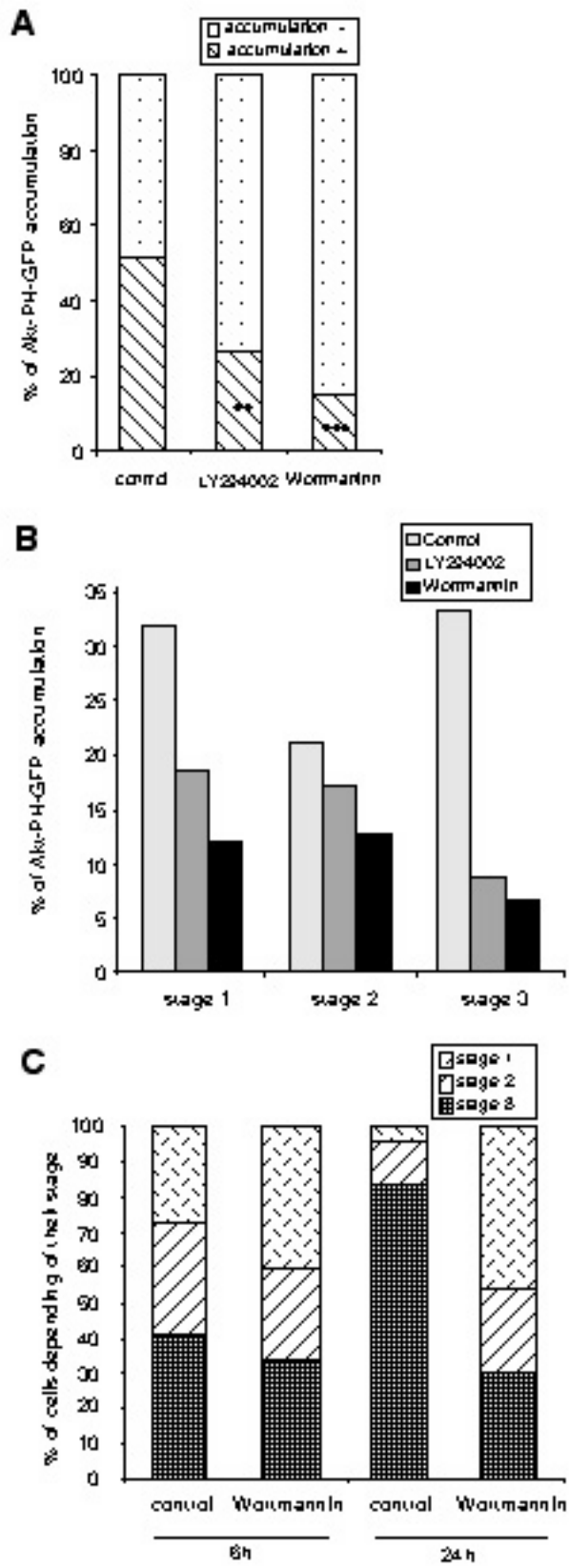


Fig. 4


A
accumulation -
accumulation +
% of Akt-PH-GFP accumulation
100
90
80
40
20
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***
contrd
LY294002
Wortmannin
B
Control
LY294002
Wortmannin
% of Akt-PH-GFP accumulation
35
30
25
20
15
10
5
0
stage 1
stage 2
stage 3
C
stage 1
stage 2
stage 3
% of cells depending of their stage
100
90
80
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60
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40
30
20
10
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Wortmannin
control
Wortmannin
6h
24 h

Fig. 5 A

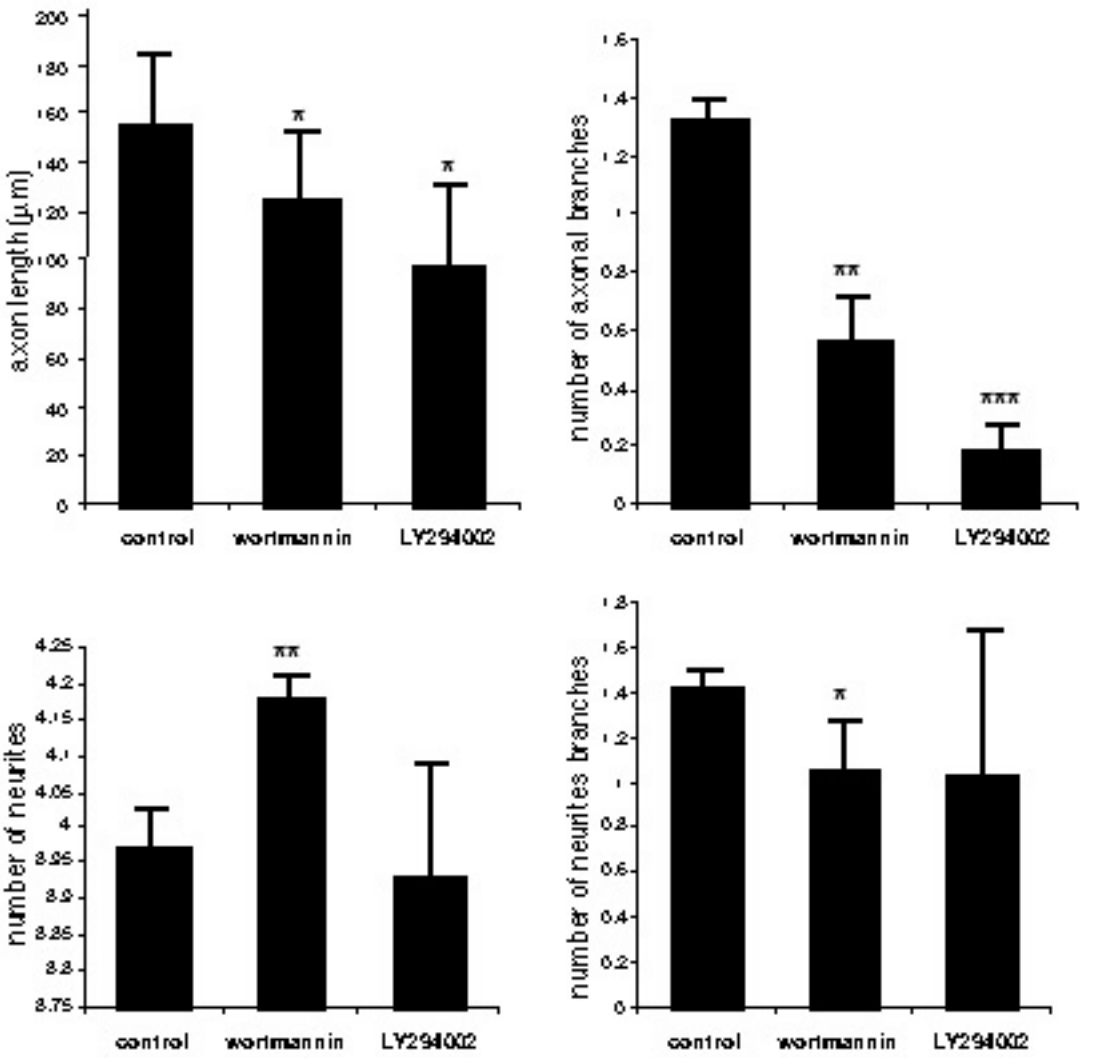
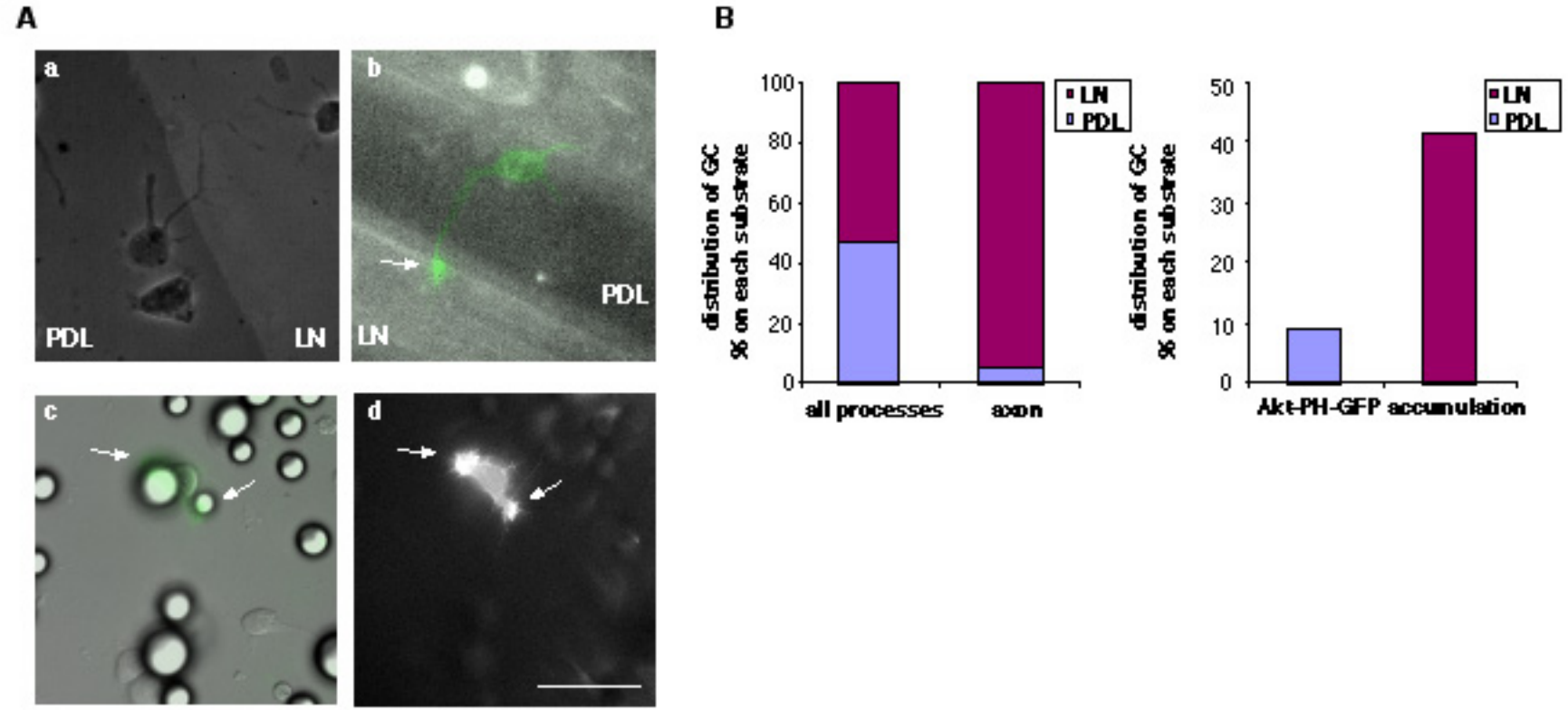

axon length (µm)
control
wortmannin
LY294002
number of axonal branches
number of neurites
number of neurites branches

Fig. 6


A
a
PDL
LN
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LN
PDL
c
d
B
distribution of GC
% on each substrate
100
80
60
40
20
0
LN
PDL
all processes
axon
distribution of GC
% on each substrate
50
40
30
20
10
0
LN
PDL
Akt-PH-GFP accumulation

Fig. 7

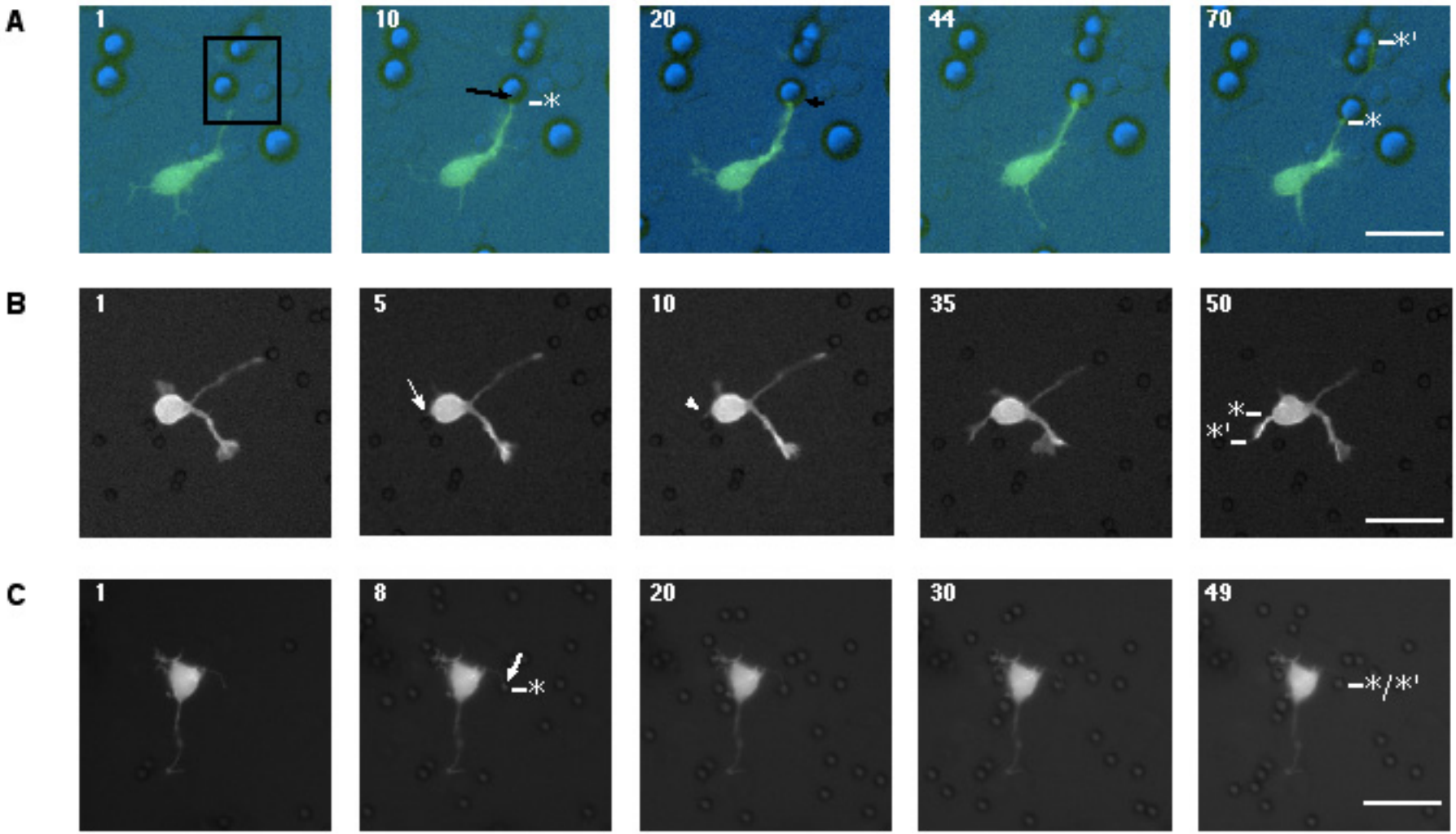


Fig. 8

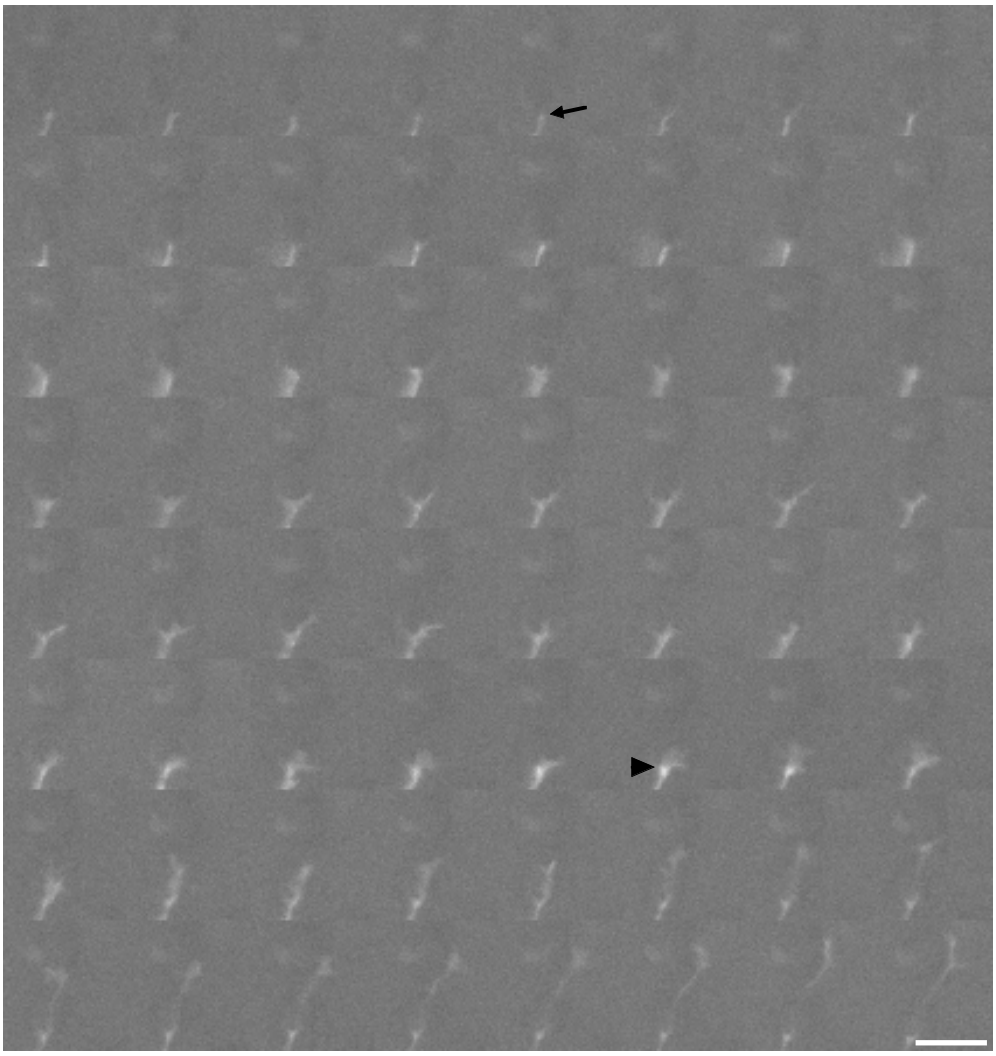


Fig. 10

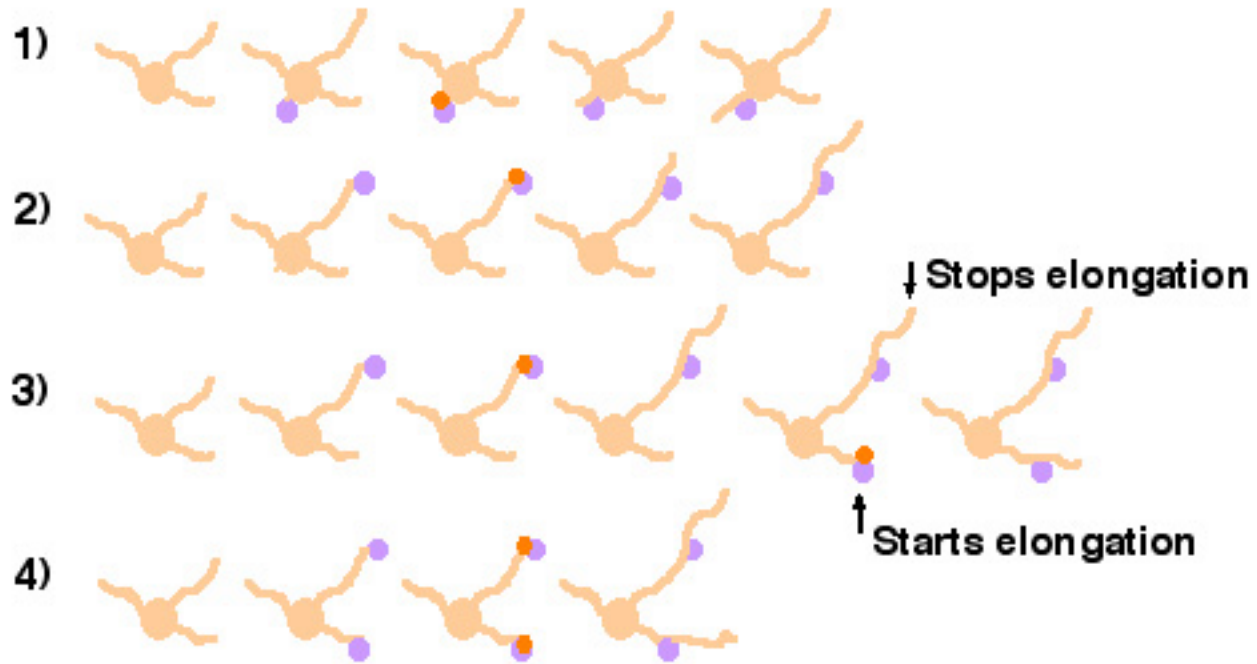
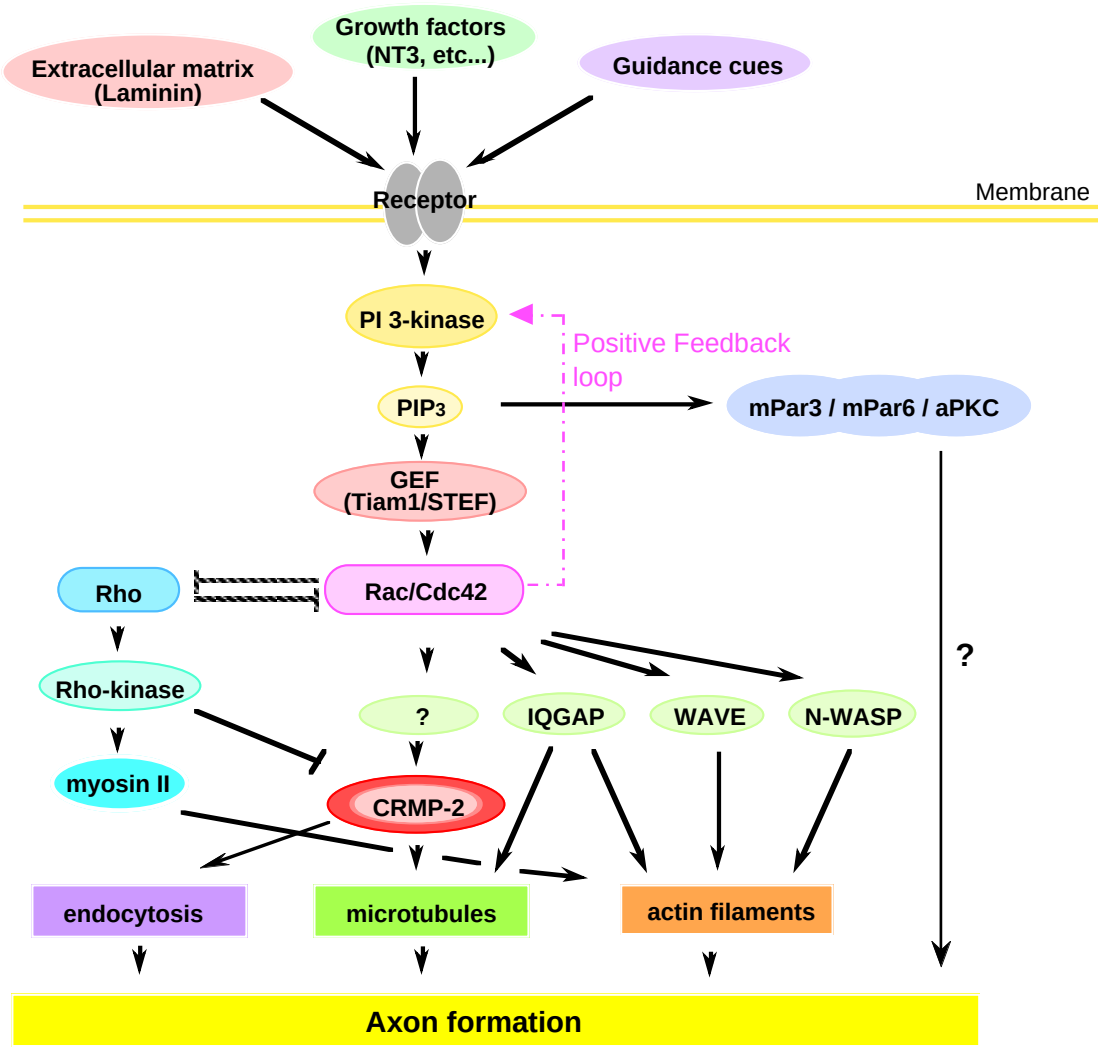


Fig. 11


Growth factors
(NT3, etc...)
Extracellular matrix
(Laminin)
Guidance cues
Receptor
Membrane
PI 3-kinase
Positive Feedback
loop
PIP3
mPar3 / mPar6 / aPKC
GEF
(Tiam1/STEF)
Rho
Rac/Cdc42
?
Rho-kinase
?
IQGAP
WAVE
N-WASP
myosin II
CRMP-2
endocytosis
microtubules
actin filaments
Axon formation