Axon Extension Occurs Independently of Centrosomal Microtubule Nucleation

Michael Stiess, *et al.*

Science **327**, 704 (2010);
DOI: 10.1126/science.1182179

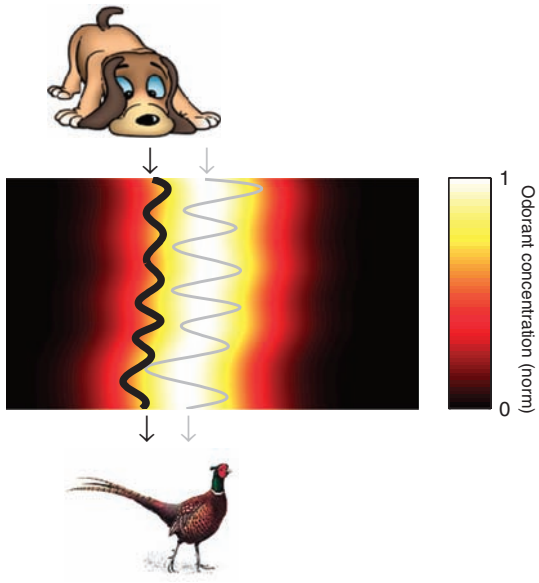


Fig. 4. Prediction for other sensory systems (olfaction). Color map, schematic odor trail; gray line, path of an organism that followed the trail's peak concentration. This strategy is typically assumed for odor-trail following (*3*). Black line, path of the same organism when using a strategy similar to that of our bats, that is, following the maximum slope of the odorant concentration (*17*). The movement jitter in this case is smaller, making the tracking smoother and therefore faster.

olfactory-tracking task indicated that *Drosophila* larvae seem to follow a trajectory between the peak and the maximum slope (*26*). Similarly, in the case of vision, we predict that when tracking large moving objects, humans would place their fovea on the object's intensity slope to optimize tracking. Finally, several recent studies have reported sensory neurons that best encode stimulus location via the maximum slope of their tuning curve (*22*, *27*–*29*), not via the peak firing rate of the tuning curve. Such coding maximizes the discriminability of the on-slope stimulus, paralleling our behavioral results, which show an optimal-localization strategy at the sensor's behavioral level.

References and Notes

1. D. Kleinfeld, E. Ahissar, M. E. Diamond, *Curr. Opin. Neurobiol.* **16**, 435 (2006).
2. K. C. Catania, *Nature* **444**, 1024 (2006).
3. J. Porter *et al.*, *Nat. Neurosci.* **10**, 27 (2007).
4. N. Ulanovsky, C. F. Moss, *Proc. Natl. Acad. Sci. U.S.A.* **105**, 8491 (2008).
5. M. E. Nelson, M. A. MacIver, *J. Comp. Physiol. A* **192**, 573 (2006).
6. W. W. Au, K. J. Benoit-Bird, *Nature* **423**, 861 (2003).
7. G. Jones, M. W. Holderied, *Proc. Biol. Sci.* **274**, 905 (2007).
8. H.-U. Schnitzler, C. F. Moss, A. Denzinger, *Trends Ecol. Evol.* **18**, 386 (2003).
9. J. A. Simmons, M. B. Fenton, M. J. O'Farrell, *Science* **203**, 16 (1979).
10. N. Suga, *J. Exp. Biol.* **146**, 277 (1989).
11. W. Metzner, *Nature* **341**, 529 (1989).
12. W. M. Masters, A. J. Moffat, J. A. Simmons, *Science* **228**, 1331 (1985).
13. K. Ghose, C. F. Moss, *J. Neurosci.* **26**, 1704 (2006).
14. N. Ulanovsky, M. B. Fenton, A. Tsoar, C. Korine, *Proc. Biol. Sci.* **271**, 1467 (2004).
15. C. Chiu, W. Xian, C. F. Moss, *Proc. Natl. Acad. Sci. U.S.A.* **105**, 13116 (2008).
16. E. K. V. Kalko, H.-U. Schnitzler, *Behav. Ecol. Sociobiol.* **33**, 415 (1993).
17. Materials and methods are available as supporting material on *Science* Online.
18. R. A. Holland, D. A. Waters, J. M. Rayner, *J. Exp. Biol.* **207**, 4361 (2004).
19. H. V. Herbert, *Z. Saugetierkd.* **50**, 141 (1985).
20. K. Ghose, C. F. Moss, *J. Acoust. Soc. Am.* **114**, 1120 (2003).
21. P. Dayan, L. F. Abbott, *Theoretical Neuroscience* (MIT Press, Cambridge, MA, 2001).
22. D. A. Butts, M. S. Goldman, *PLoS Biol.* **4**, e92 (2006).
23. A. Surlykke, K. Ghose, C. F. Moss, *J. Exp. Biol.* **212**, 1011 (2009).
24. T. Manabe, N. Suga, J. Ostwald, *Science* **200**, 339 (1978).
25. R. S. Heffner, G. Koay, H. E. Heffner, *J. Comp. Psychol.* **113**, 297 (1999).
26. M. Louis, T. Huber, R. Benton, T. P. Sakmar, L. B. Vosshall, *Nat. Neurosci.* **11**, 187 (2008).
27. N. S. Harper, D. McAlpine, *Nature* **430**, 682 (2004).
28. A. Brand, O. Behrend, T. Marquardt, D. McAlpine, B. Grothe, *Nature* **417**, 543 (2002).
29. D. McAlpine, D. Jiang, A. R. Palmer, *Nat. Neurosci.* **4**, 396 (2001).

30. We thank N. Sobel, J. Rubin, E. Simoni, A. Rubin, and N. Harper for comments on the manuscript; M. Holderied, I. Couzin, A. Arieli, E. Ahissar, and H.-U. Schnitzler for helpful discussions; and J. Barcelo for technical assistance. This study was funded by a Human Frontiers Science Program grant to N.U. and a Weizmann Institute Postdoctoral Fellowship to Y.Y.

Supporting Online Material

www.sciencemag.org/cgi/content/full/327/5966/701/DC1
Materials and Methods
SOM Text
Figs. S1 to S7
Movie S1
References

14 October 2009; accepted 23 December 2009
10.1126/science.1183310

Axon Extension Occurs Independently of Centrosomal Microtubule Nucleation

Michael Stiess,[1] Nicola Maghelli,[2] Lukas C. Kapitein,[3] Susana Gomis-Rüth,[1] Michaela Wilsch-Bräuninger,[2] Casper C. Hoogenraad,[3] Iva M. Tolić-Nørrelykke,[2] Frank Bradke[1]*

Microtubules are polymeric protein structures and components of the cytoskeleton. Their dynamic polymerization is important for diverse cellular functions. The centrosome is the classical site of microtubule nucleation and is thought to be essential for axon growth and neuronal differentiation—processes that require microtubule assembly. We found that the centrosome loses its function as a microtubule organizing center during development of rodent hippocampal neurons. Axons still extended and regenerated through acentrosomal microtubule nucleation, and axons continued to grow after laser ablation of the centrosome in early neuronal development. Thus, decentralized microtubule assembly enables axon extension and regeneration, and, after axon initiation, acentrosomal microtubule nucleation arranges the cytoskeleton, which is the source of the sophisticated morphology of neurons.

The centrosome is regarded as the primary source of microtubules in axonal and dendritic growth (*1*, *2*). It is thought that microtubules assemble at the centrosome, then are released and move along the axon through motor proteins (*1*, *3*, *4*). Furthermore, in vitro the centrosome directs axon formation in vertebrate and invertebrate neurons (*5*, *6*), but this has not been confirmed in vivo (*7*). Microtubules, however, can also assemble locally from subunits or small oligomers within the axon (*8*–*10*). Indeed, flies that lose centrosomes during development seem to develop a largely normal nervous system, where the direction of axon outgrowth appears not to be affected (*11*). Thus, the role of the centrosome and centrosomal microtubule nucleation in axon growth is controversial (*12*–*15*).

To define the role of the centrosome in microtubule nucleation during neuronal development, we first determined where microtubules are nucleated during the development of rodent hippocampal neurons. Microtubules were depolymerized with nocodazole, and the microtubule nucleation sites were examined after washout of the drug (Fig. 1A). In young neurons that had just initiated an axon [2 days in vitro (DIV)], microtubules

[1]Independent Junior Research Group Axonal Growth and Regeneration, Max Planck Institute of Neurobiology, Am Klopferspitz 18, 82152 Martinsried, Germany. [2]Max Planck Institute of Molecular Cell Biology and Genetics, Pfotenhauerstrasse 108, 01307 Dresden, Germany. [3]Department of Neuroscience, Erasmus Medical Center, Dr. Molewaterplein 50, 3015 GE Rotterdam, Netherlands.

*To whom correspondence should be addressed. E-mail: fbradke@neuro.mpg.de

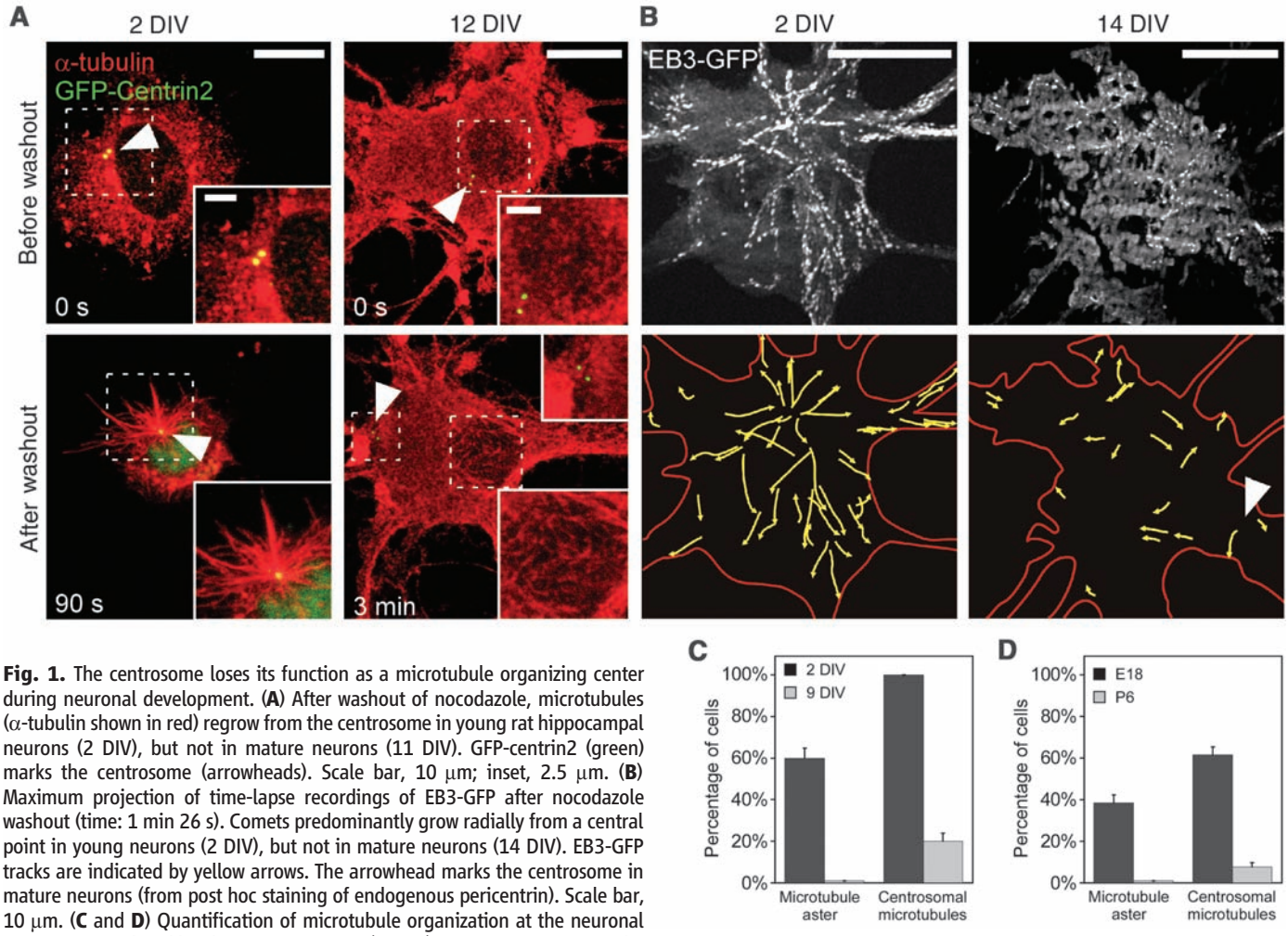


Fig. 1. The centrosome loses its function as a microtubule organizing center during neuronal development. (**A**) After washout of nocodazole, microtubules (α-tubulin shown in red) regrow from the centrosome in young rat hippocampal neurons (2 DIV), but not in mature neurons (11 DIV). GFP-centrin2 (green) marks the centrosome (arrowheads). Scale bar, 10 μm; inset, 2.5 μm. (**B**) Maximum projection of time-lapse recordings of EB3-GFP after nocodazole washout (time: 1 min 26 s). Comets predominantly grow radially from a central point in young neurons (2 DIV), but not in mature neurons (14 DIV). EB3-GFP tracks are indicated by yellow arrows. The arrowhead marks the centrosome in mature neurons (from post hoc staining of endogenous pericentrin). Scale bar, 10 μm. (**C** and **D**) Quantification of microtubule organization at the neuronal centrosome analyzed by electron microscopy (fig. S1) in neurons at 2 DIV and 9 DIV [n = 10 cells each, (C)] as well as in E18 and P6 hippocampi [n = 13 cells each, (D)]. Error bars denote SEM of a binominal distribution.

regrew at the centrosome in 83% of the neurons (n > 200 cells) (*16*). Microtubule seeds were also visible at acentrosomal sites. In mature neurons (11 to 12 DIV), microtubules nucleated randomly throughout the whole cell but did not emanate from the centrosome (n > 30 cells). In young neurons expressing the microtubule plus-end binding protein 3 (EB3) fused to green fluorescent protein (GFP) (*17*), microtubules grew from the centrosome, but also from acentrosomal sites after nocodazole washout (n = 11 cells; Fig. 1B and movie S1). In contrast, in mature neurons (14 DIV), EB3-GFP comets emerged all over the cell body (n = 14 cells; Fig. 1B and movie S2). Acentrosomal microtubule growth speed [67 ± 23 nm/s (mean ± SD); n = 83 comets] was indistinguishable from centrosomal microtubule growth speed (64 ± 24 nm/s; n = 85 comets) in young neurons, whereas the acentrosomal microtubule growth speed in mature neurons was slightly reduced (58 ± 15 nm/s; n = 435 comets).

Electron microscopy revealed structural changes at the centrosome that correlate with the loss in centrosomal activity during neuronal development. In young neurons (2 DIV), microtubules emanated from centrioles in all analyzed neurons and formed an aster-like structure in 60% of the cells (n = 10 cells; Fig. 1C and fig. S1). In contrast, in differentiated neurons (9 DIV), many microtubules were present in the cell body, but in only 20% of the neurons were the centrioles or the pericentriolar region linked to microtubules (n = 10 cells; Fig. 1C and fig. S1). We observed a similar decrease of microtubules emerging out of the pericentriolar area in vivo, comparing centrosomes in pyramidal neurons of the hippocampus at embryonic day 18 (E18) and at postnatal day 6 (P6; n = 13 cells each; Fig. 1D and fig. S1). Thus, the centrosome loses its function as a microtubule organizing center (MTOC) during neuronal development.

Immunofluorescence microscopy showed that γ-tubulin, a key protein in microtubule nucleation (*18*) that is essential for axon outgrowth (*19*), changed its intracellular localization during neuronal development (Fig. 2, A and B). At 1 DIV, when axons start to form, γ-tubulin was localized to the centrosome in all neurons, showing an intensive centrosomal staining (n = 44 cells; Fig. 2, A and B). At 4 to 5 DIV, when both axons and dendrites grow, centrosomal γ-tubulin was detected in 97% of the neurons, but the intensity was reduced by 52% (n = 49 cells; Fig. 2, A and B). In mature neurons (11 to 12 DIV), centrosomal γ-tubulin was detectable in only 42% of the neurons and the intensity was reduced by 81% (n = 43 cells; Fig. 2, A and B). However, γ-tubulin was still present in differentiated neurons and in biochemical extracts of axons (Fig. 2, C and D). Pericentrin, another component of the pericentriolar material (PCM), also decreased at the centrosome, whereas the centriolar protein centrin remained relatively constant (Fig. 2B). Thus, the microtubule nucleator γ-tubulin becomes delocalized from the centrosome during neuronal differentiation.

We next analyzed the expression levels of proteins that play a role in recruiting γ-tubulin to the centrosome. Nedd1, which is part of the γ-tubulin ring complex (γTuRC) and targets the γTuRC to the PCM (*20*, *21*), was reduced during development (Fig. 2C). Moreover, the centrosomal protein 4.1–associated protein (CPAP), a protein required for centriole duplication (*22*), strongly decreased during neuronal differentiation (Fig. 2C). Consistently, ectopically expressed GFP–γ-tubulin was recruited to the centrosome in young neurons (1 DIV) in 100% of the cells (n = 258 cells; Fig. 2E and fig. S2), whereas no

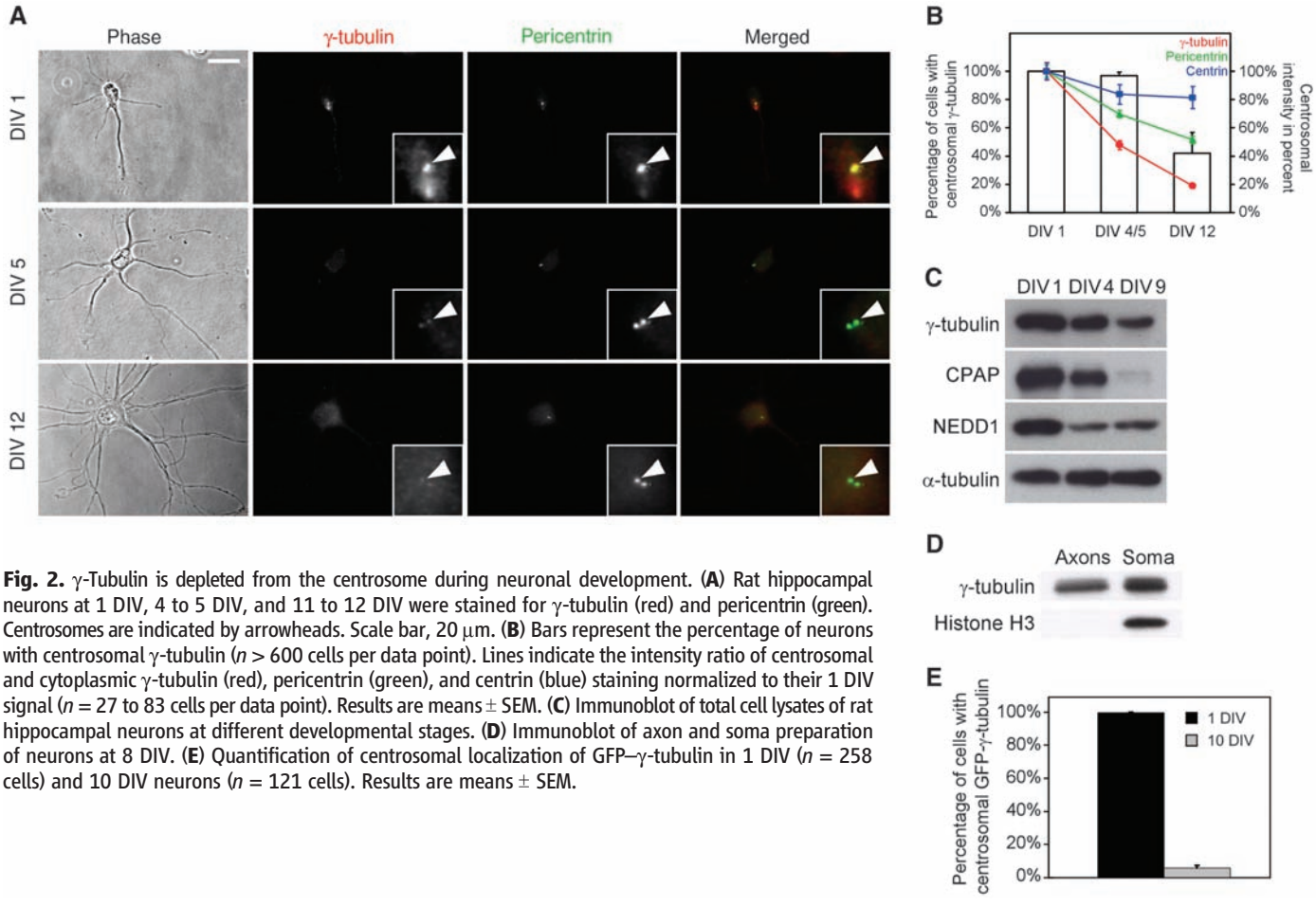


Fig. 2. γ-Tubulin is depleted from the centrosome during neuronal development. (**A**) Rat hippocampal neurons at 1 DIV, 4 to 5 DIV, and 11 to 12 DIV were stained for γ-tubulin (red) and pericentrin (green). Centrosomes are indicated by arrowheads. Scale bar, 20 μm. (**B**) Bars represent the percentage of neurons with centrosomal γ-tubulin ($n > 600$ cells per data point). Lines indicate the intensity ratio of centrosomal and cytoplasmic γ-tubulin (red), pericentrin (green), and centrin (blue) staining normalized to their 1 DIV signal ($n = 27$ to 83 cells per data point). Results are means ± SEM. (**C**) Immunoblot of total cell lysates of rat hippocampal neurons at different developmental stages. (**D**) Immunoblot of axon and soma preparation of neurons at 8 DIV. (**E**) Quantification of centrosomal localization of GFP–γ-tubulin in 1 DIV ($n = 258$ cells) and 10 DIV neurons ($n = 121$ cells). Results are means ± SEM.

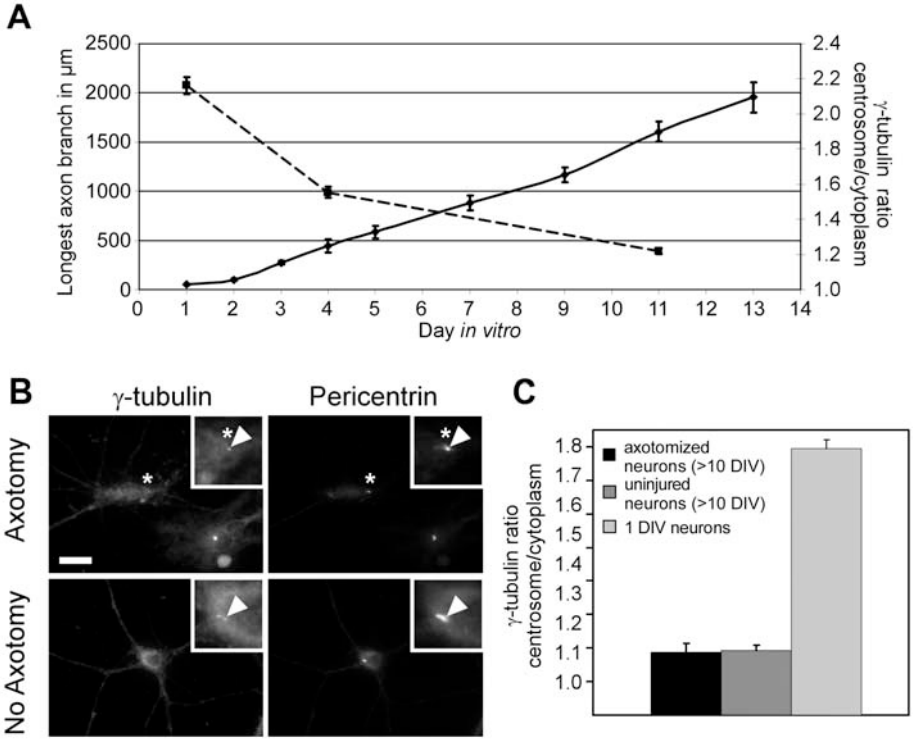
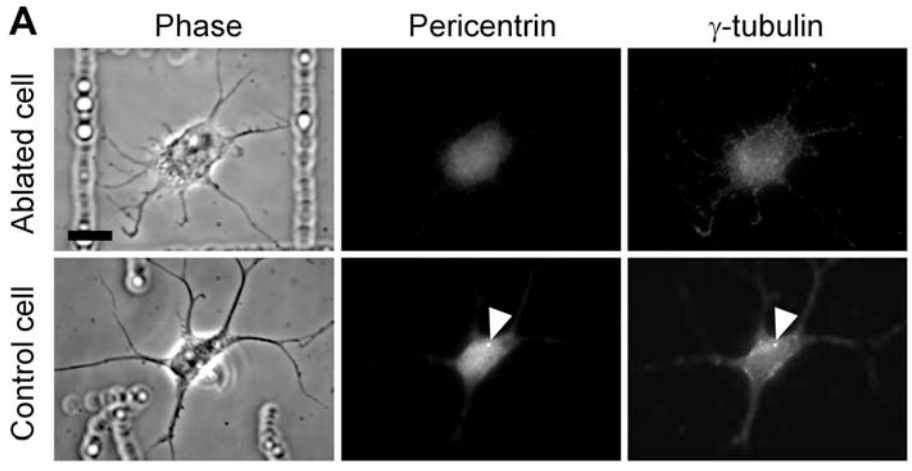


Fig. 3. Axon extension occurs in differentiated neurons in the absence of centrosomal γ-tubulin. (**A**) Growth of longest axon branch of single GFP-labeled mouse hippocampal neurons over time (black line, $n = 18$ to 52 cells per data point) and intensity ratio of centrosomal and cytoplasmic γ-tubulin staining (dashed line; from Fig. 2B). Results are means ± SEM. (**B**) Mouse hippocampal neuron 24 hours after axotomy at 14 DIV and uninjured control neuron. Arrowheads indicate centrosomes. Scale bar, 20 μm. (**C**) Quantification of intensity ratio of centrosomal and cytoplasmic γ-tubulin staining in axotomized ($n = 20$ cells), non-axotomized ($n = 244$ cells), and young neurons (1 DIV, $n = 60$ cells). Results are means ± SEM.



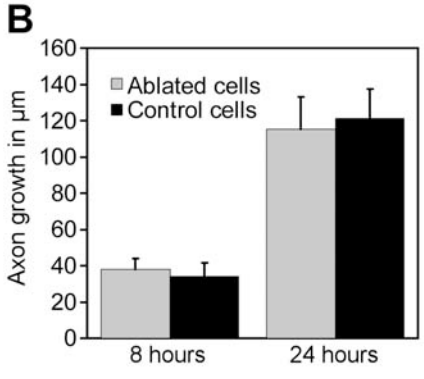


Fig. 4. Laser ablation of the centrosome in young neurons does not affect axon extension. (**A**) Ablated neuron from fig. S5 shows no centrosomal γ-tubulin and pericentrin staining. Arrowheads mark centrosomal staining in a control neuron. Scale bar, 10 μm. (**B**) Axons grow similarly 8 hours and 24 hours after ablation compared to axons of control cells (n = 12 to 52 cells). Results are means ± SEM.

specific recruitment of GFP–γ-tubulin to the centrosome was observed in differentiated neurons (10 DIV) (Fig. 2E and fig. S2). Thus, mature neurons cannot recruit γ-tubulin to centrosomes.

Because axon growth depends on microtubule polymerization (*23*), we investigated whether axon extension is affected when the centrosome is depleted of γ-tubulin and has lost its function as a MTOC. Axons continued to grow throughout all developmental stages (Fig. 3A and fig. S3). On average, axons grew 131 μm/day (2 to 4 DIV) when γ-tubulin was present at the centrosome, and 178 μm/day (9 to 13 DIV) when the centrosome was inactive (Fig. 3A). Because the axon was also branching, the total axon growth rate increased with time. Thus, the loss of centrosomal microtubule nucleation does not restrain axon growth in mature neurons. Moreover, after axon injury by a microneedle, when a large amount of microtubules was removed, axon regeneration occurred without γ-tubulin recruitment to the centrosome. When an axon grew after axotomy in neurons at 8 to 17 DIV, either from an existing dendrite or from the cut axonal stump (n = 20 cells; fig. S4) (*24*), centrosomal γ-tubulin remained at low levels similar to those seen in unlesioned cells (n = 244 cells; Fig. 3, B and C). Thus, axons extend and regenerate independently of centrosomal microtubule nucleation in mature neurons.

We next asked whether axon extension requires centrosomal microtubule nucleation in earlier stages of development, when the centrosome still functions as a MTOC. With the use of a two-photon laser ablation setup (*25*), we destroyed the centrosome, visualized by GFP-centrin2, in neurons that had just started to form an axon (2 DIV; fig. S5). Centrosomal staining of γ-tubulin, pericentrin, and centrin was not found in centrosome-ablated neurons, either after ablation or at the end of the experiment (Fig. 4A and fig. S6). Electron microscopy confirmed the expected physical destruction of the centrosome in ablated neurons (*26*) (fig. S7). In centrosome-ablated neurons, only acentrosomal microtubule regrowth occurred after nocodazole washout (n = 19; fig. S8). Live imaging of EB3-GFP showed no centrosomal but only acentrosomal microtubule assembly in ablated cells (fig. S8 and movies S3 and S4). After ablation, axon growth was followed during the subsequent 24 hours in the surviving neurons (Fig. 4B and fig. S5). The axon of centrosome-ablated neurons grew 115 μm on average (n = 12 cells), similar to control neurons (121 μm; n = 26 cells) (P = 0.81, two-tailed t test). Thus, young neurons also extend their axon without a centrosome.

Although the centrosome is necessary for cell cycle progression and neurogenesis (*27*, *28*), here we have found that the centrosome becomes dispensable for axon extension and regeneration. Neuronal differentiation requires sophisticated architectural changes that may be incompatible with a large microtubule network emanating from a focal point. Thus, acentrosomal microtubule nucleation may be a key feature during differentiation of both neuronal and non-neuronal cells (*29*, *30*). Dismantling the centrosome and decentralizing microtubule nucleation may be essential to enable axon branching, dendrite formation, and spine generation (*17*).

References and Notes

1. P. W. Baas, A. Karabay, L. Qiang, *Trends Cell Biol.* **15**, 518 (2005).
2. H. R. Higginbotham, J. G. Gleeson, *Trends Neurosci.* **30**, 276 (2007).
3. L. Wang, A. Brown, *Curr. Biol.* **12**, 1496 (2002).
4. F. J. Ahmad, C. J. Echeverri, R. B. Vallee, P. W. Baas, *J. Cell Biol.* **140**, 391 (1998).
5. F. C. de Anda *et al.*, *Nature* **436**, 704 (2005).
6. J. F. Zmuda, R. J. Rivas, *Cell Motil. Cytoskeleton* **41**, 18 (1998).
7. F. R. Zolessi, L. Poggi, C. J. Wilkinson, C. B. Chien, W. A. Harris, *Neural Develop.* **1**, 2 (2006).
8. Y. Ma, D. Shakiryanova, I. Vardya, S. V. Popov, *Curr. Biol.* **14**, 725 (2004).
9. S. Terada, M. Kinjo, N. Hirokawa, *Cell* **103**, 141 (2000).
10. T. Kimura, H. Watanabe, A. Iwamatsu, K. Kaibuchi, *J. Neurochem.* **93**, 1371 (2005).
11. R. Basto *et al.*, *Cell* **125**, 1375 (2006).
12. N. Arimura, K. Kaibuchi, *Nat. Rev. Neurosci.* **8**, 194 (2007).
13. R. Li, G. G. Gundersen, *Nat. Rev. Mol. Cell Biol.* **9**, 860 (2008).
14. H. Witte, F. Bradke, *Curr. Opin. Neurobiol.* **18**, 479 (2008).
15. C. Conde, A. Cáceres, *Nat. Rev. Neurosci.* **10**, 319 (2009).
16. W. Yu, V. E. Centonze, F. J. Ahmad, P. W. Baas, *J. Cell Biol.* **122**, 349 (1993).
17. J. Jaworski *et al.*, *Neuron* **61**, 85 (2009).
18. C. Wiese, Y. Zheng, *J. Cell Sci.* **119**, 4143 (2006).
19. F. J. Ahmad, H. C. Joshi, V. E. Centonze, P. W. Baas, *Neuron* **12**, 271 (1994).
20. J. Lüders, U. K. Patel, T. Stearns, *Nat. Cell Biol.* **8**, 137 (2005).
21. L. Haren *et al.*, *J. Cell Biol.* **172**, 505 (2006).
22. C. J. Tang, R. H. Fu, K. S. Wu, W. B. Hsu, T. K. Tang, *Nat. Cell Biol.* **11**, 825 (2009).
23. E. Tanaka, T. Ho, M. W. Kirschner, *J. Cell Biol.* **128**, 139 (1995).
24. S. Gomis-Rüth, C. J. Wierenga, F. Bradke, *Curr. Biol.* **18**, 992 (2008).
25. N. Maghelli, I. M. Tolić-Nørrelykke, *J. Biophotonics* **1**, 299 (2008).
26. A. Khodjakov, R. W. Cole, B. R. Oakley, C. L. Rieder, *Curr. Biol.* **10**, 59 (2000).
27. S. Doxsey, D. McCollum, W. Theurkauf, *Annu. Rev. Cell Dev. Biol.* **21**, 411 (2005).
28. X. Wang *et al.*, *Nature* **461**, 947 (2009).
29. J. Lüders, T. Stearns, *Nat. Rev. Mol. Cell Biol.* **8**, 161 (2007).
30. F. Bartolini, G. G. Gundersen, *J. Cell Sci.* **119**, 4155 (2006).
31. We thank L. Meyn, N. Keijzer, and M. Kuijpers for preparing hippocampal neurons; M. Braun for electron microscopy; T. Gollisch and D. Neukirchen for critically reading the manuscript; W.B. Huttner for his support; P. Gordon-Weeks, R. Klein, J. Kleylein-Sohn, E. Nigg, O. Stemmann, and G. Tavosanis for discussions; and A. Khodjakov, M. Distel, R. Köster, E. Nigg, and J. Salisbury for sharing antibodies and plasmids. Supported by the Erasmus Medical Center and the Netherlands Organization for Scientific Research (L.C.K.), the Netherlands Organization for Health Research and Development, the European Science Foundation (ESF-EURYI) (C.C.H.), a Career Development Award from the Human Frontier Science Program (F.B. and C.C.H.), the Max Planck Society, the International Foundation for Research in Paraplegia, and the Deutsche Forschungsgemeinschaft (F.B.).

Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1182179/DC1
Materials and Methods
Figs. S1 to S8
Movies S1 to S4
References

18 September 2009; accepted 9 December 2009
Published online 7 January 2010;
10.1126/science.1182179
Include this information when citing this paper.