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Imaging neurite development of adult-born granule cells

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SUMMARY

Neural stem/progenitor cells (NSPCs) generate new neurons throughout life in the mammalian hippocampus. Newborn granule cells mature over several weeks to functionally integrate into the pre-existing neural circuitry. Even though an increasing number of genes that regulate neuronal polarization and neurite extension have been identified, the cellular mechanisms underlying the extension of neurites arising from newborn granule cells remain largely unknown. This is mainly because of the current lack of longitudinal observations of neurite growth within the endogenous niche. Here we used a novel slice culture system of the adult mouse hippocampal formation combined with *in vivo* retroviral labeling of newborn neurons and longitudinal confocal imaging to analyze the mode and velocity of neurite growth extending from immature granule cells. Using this approach we show that dendritic processes show a linear growth pattern with a speed of $2.19 \pm 0.2 \mu\text{m}$ per hour, revealing a much faster growth dynamic than expected by snapshot-based *in vivo* time series. Thus, we here identified the growth pattern of neurites extending from newborn neurons within their niche and describe a novel technology that will be useful to monitor neuritic growth in physiological and disease states that are associated with altered dendritic morphology, such as rodent models of epilepsy.

KEY WORDS: Dendrite, Granule cell, Hippocampus, Neurogenesis, Slice culture, Mouse

INTRODUCTION

Adult hippocampal neurogenesis is crucial in certain forms of hippocampus-dependent learning and memory and has been associated with several neuropsychiatric diseases: among others age-dependent cognitive decline, major depression and epilepsy (Zhao et al., 2008). In contrast to embryonic or early postnatal development, newborn granule cells in the adult brain have to integrate into a fully mature and functioning neural circuitry: the hippocampal dentate gyrus (DG). Thus, a number of intrinsic mechanisms and extrinsic cues need to regulate the maturation and final integration of newborn neurons in the DG circuitry (Ming and Song, 2011). After the initial division of a neural stem/progenitor cell (NSPC) the integration of a newborn granule cell requires several weeks: the extension of dendritic processes starts 2–5 days after cell birth, followed by rapid growth of the main apical dendrite, and the formation of the first glutamatergic, synaptic connections ~16 days after the cell is born (Zhao et al., 2006). Dendrites and their spines continue to mature over the next 2 to 3 weeks; a time that is considered to represent a crucial period for integration and survival of new neurons and that is also characterized by heightened excitability of newborn granule cells (Schmidt-Hieber et al., 2004; Wang et al., 2005; Ge et al., 2007; Marin-Burgin et al., 2012). Approximately 6–8 weeks after a cell is born the morphological and electrophysiological maturation process appears to be finished, with newborn granule cells becoming almost indistinguishable from granule cells born during embryonic or early postnatal development (Laplagne et al., 2006).

Previous studies have identified the requirement of several genes/pathways (e.g. DISC1, CDK5), transcriptional programs (e.g.

pCREB-mediated signaling), and neurotransmitter signaling (e.g. GABA) for proper neuronal development and targeted dendritic growth (Ge et al., 2006; Duan et al., 2007; Jessberger et al., 2008; Jagasia et al., 2009). However, the cellular dynamics and details of neurite growth remain unknown owing to the current lack of longitudinal observations of neuronal maturation processes of newborn neurons within their endogenous niche.

Here, we used a modified slice culture system of the adult hippocampal formation in combination with virus-mediated labeling of newborn cells in mouse, allowing us to visualize growth of neurites extending from newborn granule cells over several days using confocal imaging.

MATERIALS AND METHODS

Animals and retroviral labeling

All animal experiments were done according to the guidelines of, and were approved by, the veterinary office of the Canton of Zürich, Switzerland. For all experiments 6- to 8-week-old female C57Bl/6 mice (Janvier) or 6- to 8-week-old NestinGFP mice (NesGFP) were used (Yamaguchi et al., 2000). Retroviral vectors based on a murine Moloney leukemia virus expressing red fluorescent protein (RFP) under the control of the chicken β -actin (CAG) promoter were produced (CAG-RFP) and injected as described previously (Jessberger et al., 2008). Animals were injected 2 days before preparing samples for slice cultures ($n=10$). For timecourse analyses of neurite growth *in vivo*, animals were injected with CAG-RFP retrovirus and killed 2, 3, 4, 5, 6, 7, 8 and 9 days after viral injection ($n=3$ for each time point).

Slice cultures

Two days after viral labeling of newborn cells, mice were deeply anesthetized using pentobarbital and decapitated. The brain was quickly removed and transferred to carbogen-bubbled (95% O₂, 5% CO₂) iced modified Gey's balanced salt solution (mGBSS; 1.5 mM CaCl₂, 4.9 mM KCl, 0.2 mM KH₂PO₄, 11 mM MgCl₂, 125 mM NaCl, 2.7 mM NaHCO₃, 0.8 mM Na₂HPO₄, 22 mM Na-HEPES, 5 mM glucose). Icing was reached by freezing to -80°C with vigorous shaking every few minutes for about half an hour. The brains were allowed to equilibrate to the temperature and the medium for 10 minutes before hemispheres were taken out and processed for cutting. To reach the required cutting angle, a piece of the cortex was removed, cutting from dorsolateral to ventromedial in an angle of about 70° (Fig. 1A) (Leutgeb et al., 2003). Then the brain hemisphere was glued to the tissue platform of a vibratome (Leica VT 1000M) with the

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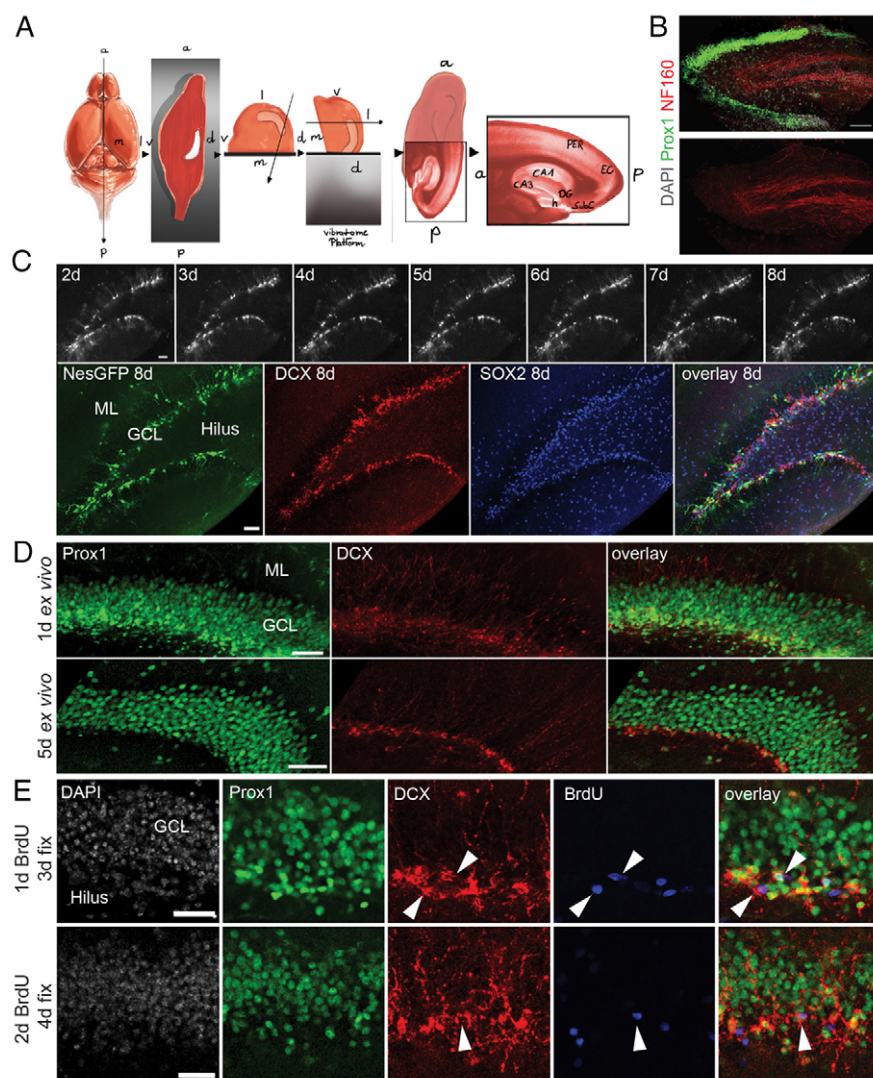


Fig. 1. Slice culture of the adult mouse hippocampal formation.

(A) Brain preparation for tissue sectioning of the hippocampal formation. (B) Visualization of the axonal marker NF160 immediately after sectioning reveals the parallel cutting plane. The upper panel shows a merged image of PROX1 (green) and NF160 (red); the lower panel shows single channel for NF160. Nuclei were counterstained with DAPI (gray). (C) The upper row of images shows repeated imaging of NesGFP-expressing NSPCs (green) from 2 to 8 days in culture. The lower row shows analyses of the same slice culture as shown above together with DCX (red) and SOX2 (blue) expression. (D) Stable expression of PROX1 (green) between 1 day in culture (upper row) and 5 days in culture (lower row) together with DCX (red). (E) Images of slices that were labeled with BrdU and analyzed for BrdU (blue), DCX (red) and PROX1 (green) co-expression. BrdU was applied to the medium as indicated and slices were analyzed at different time points thereafter (fix). Nuclei were counterstained with DAPI (gray). Arrowheads point to BrdU/DCX/PROX1 labeled cells. Scale bars: 100 μ m in B; 50 μ m in C,D; 20 μ m in E. a, anterior; d, dorsal; EC, entorhinal cortex; l, lateral; m, medial; ML, molecular layer; p, posterior; PER, perirhinal cortex; SubC, subiculum; v, ventral.

created cutting plane using Vetbond (3M) tissue adhesive. The vibratome tissue container was filled with carbogen-bubbled iced mGBSS. Brain sections of 350 μ m were taken horizontally (using high amplitude and low speed), which resembles cutting perpendicular to the septotemporal axis of the DG. After the full hemisphere was sectioned, the sections were trimmed to contain the hippocampus and entorhinal cortex only and transferred to microwell inserts (Millipore) in six-well dishes (NUNC) containing 1 ml of culturing medium per well (see below). Up to three sections were transferred onto one insert. Slices were kept at 34°C and in 5% CO₂; a third of the medium was replaced with fresh medium every day. For the first 3 days, we used medium containing 25% horse serum, 50% Eagles Basal essential media (BME), 25% Earle's Balanced Salt Solution (EBSS), 25 mM Na-Hepes, 0.5 mM L-glutamine, 28 mM glucose, 1 \times Penicillin/Streptomycin/Fungizone (PSF). From day 4 onwards, a medium with reduced serum amounts was used: 5% horse serum, 20% BME, 65% modified EBSS (1.8 mM CaCl₂, 2 mM MgSO₄, 52.5 mM NaCl, 30.9 mM NaHCO₃, 0.9 mM NaH₂PO₄), successively replacing the high serum medium, as a third of it was replaced with fresh medium every day as before. For bromodeoxyuridine (BrdU, Sigma) labeling slices were incubated with 0.3 mM BrdU for 1 hour and slices were fixed at the indicated time points.

Light microscopy imaging and image analyses

For time-lapse imaging, a Zeiss LSM 510 NLO Meta point scanning laser confocal microscope was used. Images were acquired using a 10 \times (0.3 NA) Ph1 Plan-Neofluar and a 20 \times (0.8 NA) Plan-Neofluar and a 543 nm HeNe

laser was used for excitation of RFP. Imaging was performed every 24 hours for the time of culturing. To mount the slice cultures to the microscope, a single well of a six-well plate was cut out and part of the bottom was replaced by a coverslip. In the well, a Millicell cell culture insert (Millipore) was placed. Images were acquired through glass, medium and the insert membrane at a resolution of 2048 \times 2048 pixels as a single optical slice with a slightly opened pinhole to gain the necessary depth of field. The pinhole was typically opened to 450 μ m diameter. This results in an optical section with a thickness of about 20 μ m. This was done to minimize stress by light to the cultured slices. For the time of microscopy, the slice cultures were removed from the incubator and transported to the microscope in a prewarmed container. As the microscope stage was not temperature-controlled, the time of imaging was kept as short as possible for each imaging session. Typically, a set of slices was mounted to the microscope for about 5 minutes (total time before slices were transferred back into the incubator was about 30 minutes).

The dendritic length was measured using ImageJ (NIH) with the NeuronJ plugin. For the *in vivo* timecourse, three animals per time point (24 animals in total) were injected and in total 188 cells measured (for each time point 23, 20, 21, 16, 21, 26, 30 and 31 cells, respectively). Z-axis extension of dendrites was below 20 μ m (2 days, 11.3 \pm 0.9 μ m, n =29 cells analyzed; 6 days, 15.5 \pm 0.9 μ m, n =36; 9 days, 15.9 \pm 1.0 μ m, n =34).

For the slice cultures, n =23 cells from ten animals were tracked and measured over several days. All numerical analyses were performed using Excel (Microsoft) or Prism (GraphPad).

Immunohistochemistry

Tissue was prepared as described previously (Knobloch et al., 2013). Primary antibodies used were goat α -DCX (1:250; Santa Cruz), chicken α -GFP (1:500; Aves), mouse α -NeuN (1:250; Millipore), rat α -BrdU (1:500; Abcam), mouse α -NF160 (1:250; Sigma), and rabbit α -Prox1 (1:500; Millipore). Donkey-derived secondary antibodies (Jackson Laboratory) were applied 1:250 at room temperature for 1–2 hours. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma). For staining of slice cultures, slices were fixed for 1 hour in 4°C 4% paraformaldehyde. Higher concentrations of Triton X-100 (0.4%) were used and incubation steps were extended by a factor of two given the thickness of the cultured slices. After staining, slices were mounted to object slides, covered in 10% polyvinyl alcohol (PVA, Sigma) and 2.5% 1,4-diazabicyclo[2.2.2]octane (DABCO,

Sigma) in 25% glycerol (Sigma) and coverslipped using spacers made from two layers of paper tape to avoid pressure from the coverslip.

Statistical analysis

All statistical analyses were performed using Excel (Microsoft) with appropriate add-ons or SPSS Statistics (SPSS). For comparisons of averages between two groups, unpaired Student's *t*-test was used. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Extended slice culture imaging of the adult hippocampal formation

Observing the growth of neurites extending from newborn granule cells *in vivo* in their endogenous adult niche is technically challenging. To obtain a system allowing repeated light microscopy imaging of newborn cells in their adult niche over several days, we modified a previously described protocol that had established long-term culturing of the hippocampal formation derived from adult rats (Leutgeb et al., 2003). Sections of the hippocampal formation were prepared

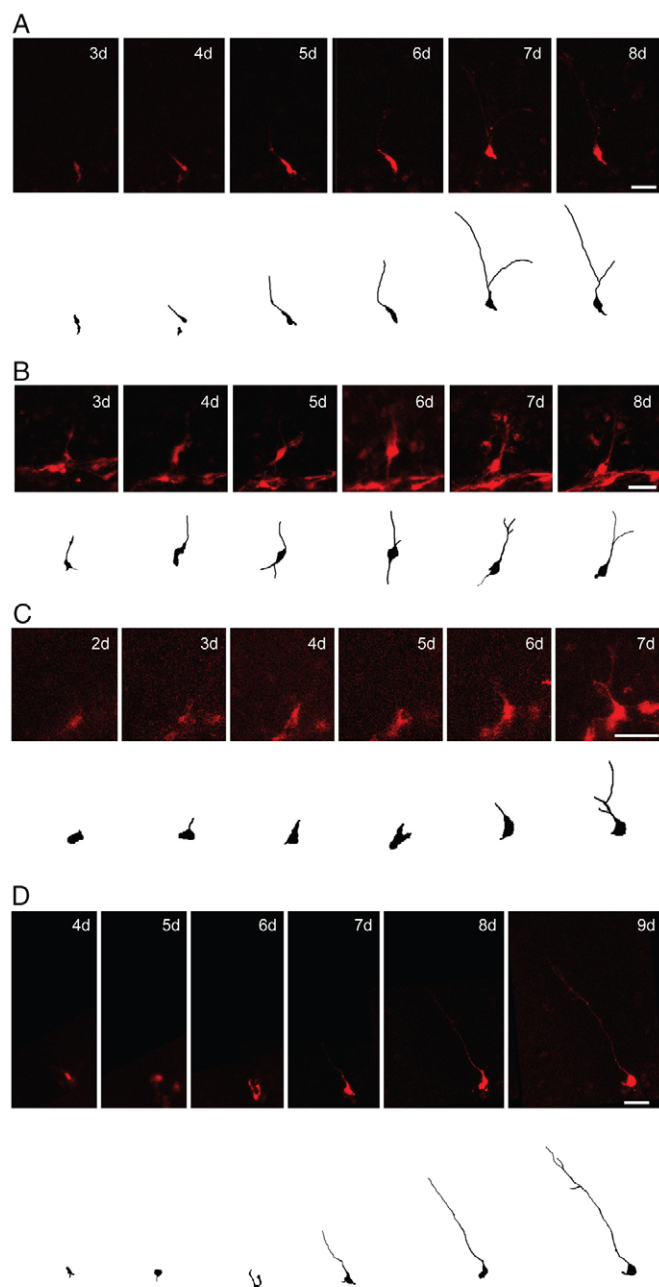


Fig. 2. Imaging of dendritic growth in individual cells. (A–D) Examples of retrovirus-labeled cells expressing RFP (red) in adult DG slice cultures. The top panels show the acquired images at the indicated time points, with a camera lucida drawing shown below. Scale bars: 20 μ m.

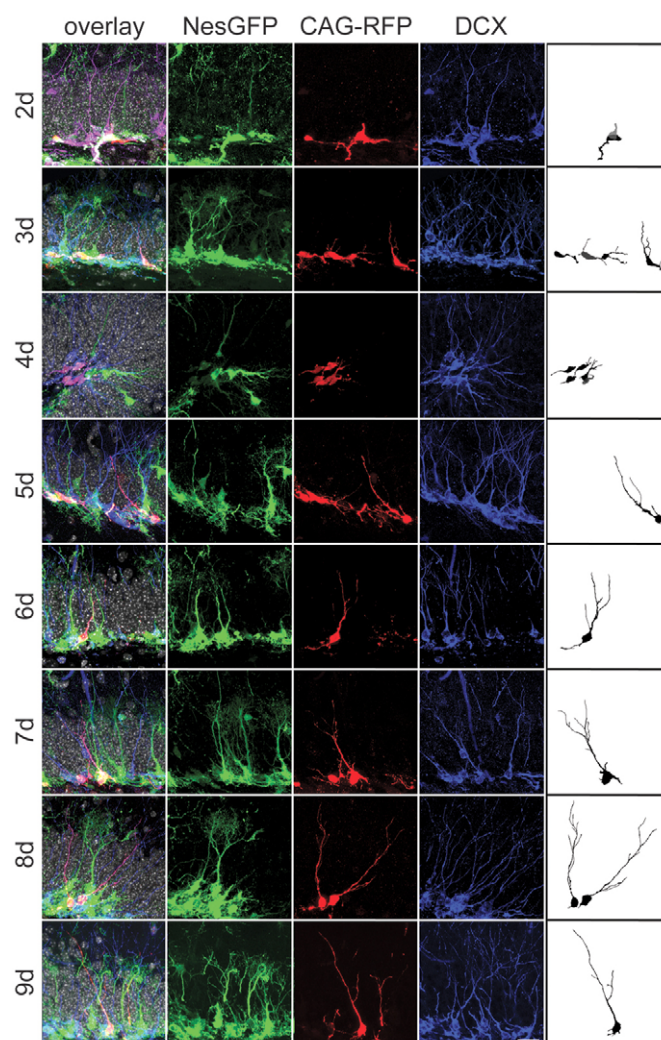


Fig. 3. Dendritic growth of retrovirus-labeled cells *in vivo*.

Representative images of newborn cells labeled with an RFP-expressing retrovirus with the age analyzed indicated on the left. Shown are overlay images and individual channels for NesGFP (green), RFP (red) and DCX (blue). Nuclei were counterstained with DAPI (gray). Scale bar: 20 μ m.

following a cortical cut from dorsolateral to ventromedial in an angle of about 70°, aiming to preserve afferent and efferent structures into and out of the adult DG (Fig. 1A). Slices were cultured on semi-porous membranes at a medium-air interface according to a method developed by Stoppini and colleagues (Stoppini et al., 1991).

We used several measures to test the structural integrity of the obtained slices utilizing immunohistochemical analyses at different time points and by repeated confocal imaging. Confirming the relative preservation of mossy fiber axons we found a substantial number of neurofilament 160 (NF160)-labeled axons extending through hilar regions that remained stable over extended times *in vitro* (Fig. 1B and data not shown). Furthermore, the number and morphology of transgenically labeled NSPCs expressing NestinGFP (NesGFP) were stable over several days (Fig. 1C and data not shown), which was also reflected by a substantial number of cells expressing Doublecortin (DCX) (Fig. 1C) (Couillard-Despres et al., 2005). In addition, the overall integrity of the granule cell layer (GCL), as measured by expression of the homeobox transcription factor PROX1, which is expressed in immature but also fully mature granule cells, remained intact for up to 8 days (Fig. 1D) (Karalay et al., 2011). Furthermore, slice cultures generated from young adult mice were still permissive of ongoing neurogenesis as measured by BrdU labeling in combination with DCX expression (Fig. 1E), similar to the situation observed in slice cultures generated from early postnatal hippocampal tissue (Raineteau et al., 2004), and further confirming the integrity of the hippocampal slices obtained from adult tissue.

Repeated imaging of neurites extending from newborn granule cells

After establishing the slice culture system, we next sought to analyze the growth of neurites extending from newborn granule cells. To visualize newborn neurons and their processes, we used a retroviral approach allowing the selective labeling of proliferating, neurogenic cells and their neuronal progeny *in vivo* (Zhao, 2008). Two days after stereotactic injection of retroviruses encoding for RFP into the DG, animals were killed and slices were obtained. We then used repeated confocal imaging over the course of up to 9 days after viral injections and measured dendritic length (Fig. 2A–D). This approach allowed us to follow individual cells over time and to analyze the speed and pattern of neurite extension. Initial

processes extended into different directions relative to the hilar/GCL border and overall the growth of dendrites showed considerable variability, similar to *in vivo* observations based on DCX expression and thymidine labeling (Plümpe et al., 2006). However, all neurons showed the clear extension of an apical dendrite at ~4 days in culture (translating into 125.1±8.7 hours after viral injection) that continued to grow over time (Fig. 2). Interestingly, all imaged neurons showed a linear growth of the apical dendrite even though analyses with higher temporal resolution will be required in the future (Fig. 2, Fig. 4B, supplementary material Movie 1). Using longitudinal imaging of newborn neurons the velocity of neurite extension could be determined and showed a growth rate of 2.19±0.2 µm per hour.

Dendritic growth in slice cultures is comparable to growth characteristics within the intact brain

We next compared dendritic growth of granule cells visualized in the slice culture system with snapshot-based, timecourse analyses of virally labeled newborn granule cells in the intact DG. Animals were injected with RFP-expressing retrovirus and killed at 24 hour intervals between 2 and 9 days after viral injection (Fig. 3). We then analyzed dendritic length at each time point and compared these numbers to the values obtained by repeated imaging of individual cells in the slice cultures. By plotting these data against time, we found that the values of total dendritic length were comparable between the *in vivo* snapshot-based data and the results obtained in the slice cultures (Fig. 4A,B). However, we found differences in the single-cell-measured velocity of neurite growth in the slice culture system compared with the calculated extension of neurites using the snapshot samples: when we used linear regression to measure the averaged speed of neurite extension in the timecourse series (obviously not being able to do these calculations for single, defined cells) we found a calculated speed of 0.69 µm/hour; a value much lower compared with the single-cell data obtained by repeated imaging of the slice cultures, where we found a growth rate of 2.19±0.2 µm/hour (see above). Neurite extension has a highly variable onset in newborn granule cells, which is supported by previous data that used DCX expression to measure the shape and extension of dendritic processes in fixed tissue (Plümpe et al., 2006). A linear regression on the averages of dendritic length of the *in vivo* data omits this phenomenon. Thus, we calculated the mean onset

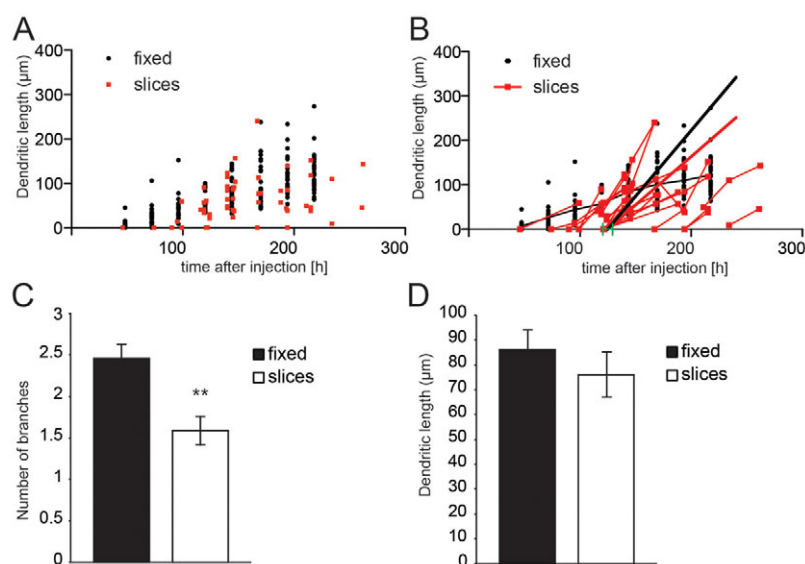


Fig. 4. Dendritic growth pattern in slice cultures versus snapshot-based *in vivo* data. (A) Dendritic length over time in fixed samples (black dots) and for cells analyzed in slice cultures (red squares), indicating the overall similarity between *in vivo* and slice culture growth potential. (B) Speed of dendritic growth for imaged individual cells (connected with red lines). Note that slice culture analyses allow for defining the actual starting point of dendritic extension (zero value). For *in vivo* cells, the means are indicated by the connecting black line. Solid strokes indicate the linear slope for neurite extension for the observed values in the slice cultures and the calculated values for the *in vivo* data after correction for the mean onset time before the start of neurite extension. (C) Dendritic branching is reduced in slice cultures compared with the *in vivo* situation 6 days after viral injection. (D) In contrast to the dendritic complexity, the net growth of neurites is comparable between slice cultures and *in vivo* conditions 6 days after labeling newborn cells using a retrovirus-based approach. ** $P < 0.01$.

of neurite extension observed in slice cultures (which was 125.1 ± 8.7 hours after viral injection) and recalculated the growth speed *in vivo* by subtracting this newly obtained value. Strikingly, we found that after this 'simulation' the growth speed between slices and *in vivo* became highly similar (2.19 ± 0.2 $\mu\text{m}/\text{hour}$ for longitudinally observed neurites in the slice cultures and calculated 2.74 $\mu\text{m}/\text{hour}$ for *in vivo* snapshot observations). These findings indicate a high similarity between the slice culture conditions and the *in vivo* situation and strongly support the validity of our approach.

However, neuronal maturation was not identical between slice cultures and the *in vivo* conditions as we found that neurites extending in the slices showed slightly reduced branching compared with the cells analyzed in fixed samples 6 days after viral injections, even though the total dendritic length was not reduced (Fig. 4C,D) (branching *in vivo*: 2.45 ± 0.17 , slice culture: 1.58 ± 0.17 , $P < 0.01$; dendritic length *in vivo*: 85.9 ± 8.05 , slice culture: 76.04 ± 9.04 , $P > 0.05$). Thus, the slice culture approach does not reflect completely and for all aspects the growth pattern *in vivo*, similar to what has been described for the widely used slice culture system of the early postnatal hippocampus, which shows a number of substantial differences from the *in vivo* situation but still turned out to be an extremely valuable tool (Heimrich and Frotscher, 1991; Gähwiler et al., 1997).

The slice-culture-based approach of the adult neurogenic niche presented here offers a novel experimental setup that allows investigation of the dynamic processes that underlie neuronal maturation and dendritic integration in the context of adult hippocampal neurogenesis using genetic and pharmacological manipulations.

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Competing interests statement

The authors declare no competing financial interests.

Author contributions

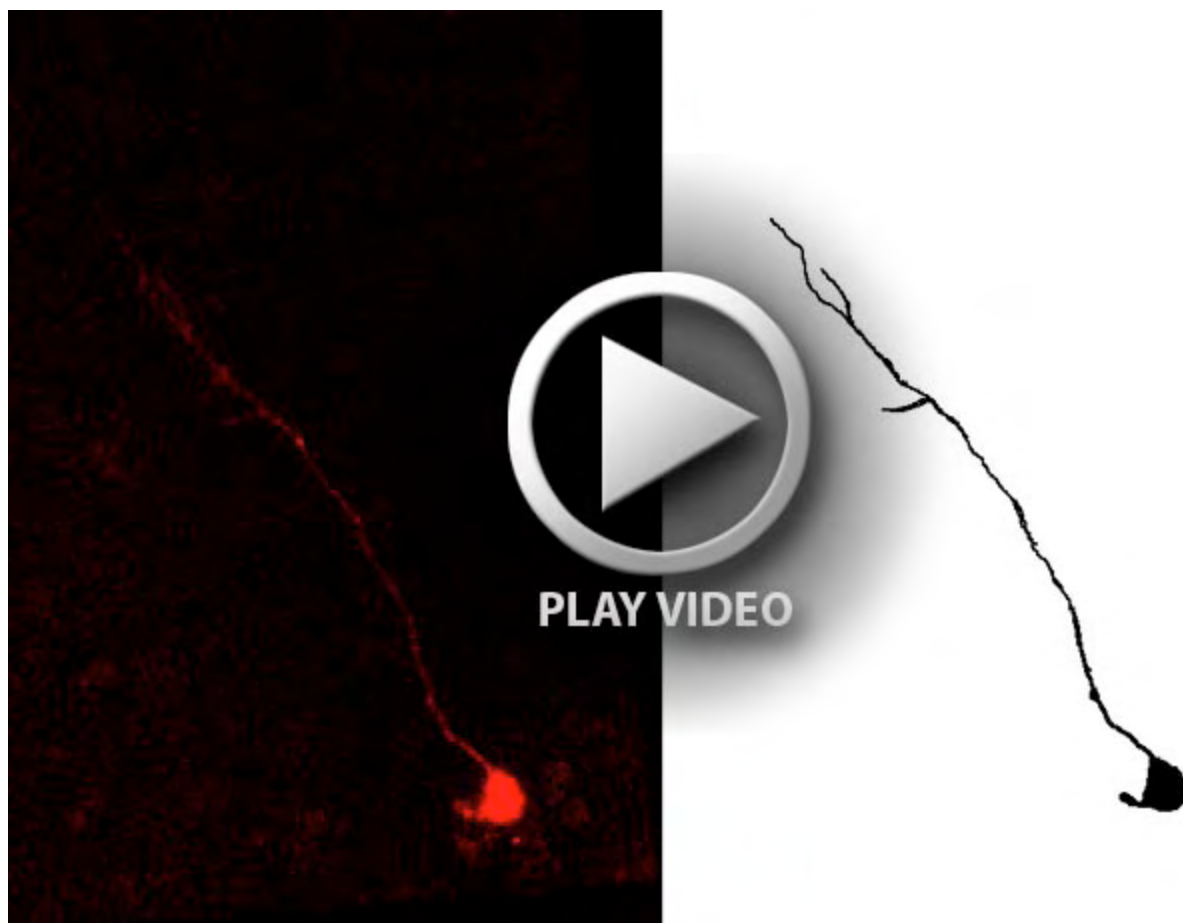
F.B.K.B. performed experiments, analyzed data and co-wrote the manuscript; O.B. performed experiments and analyzed data; S.J. developed the concept, performed experiments and wrote the paper.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.091249/-/DC1>

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Movie 1. Repeated confocal imaging of adult-born neuron extending neurites in hippocampal slice cultures. The movie starts 4 days after retroviral injection in vivo. The culture is imaged every 24 hours for 6 days. The right panel shows a digital reconstruction.