In vivo imaging of dendritic pruning in dentate granule cells

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We longitudinally imaged the developing dendrites of adult-born mouse dentate granule cells (DGCs) in vivo and found that they underwent over-branching and pruning. Exposure to an enriched environment and constraint of dendritic growth by disrupting Wnt signaling led to increased branch addition and accelerated growth, which were, however, counteracted by earlier and more extensive pruning. Our results indicate that pruning is regulated in a homeostatic fashion to oppose excessive branching and promote a similar dendrite structure in DGCs.

In the rodent brain the dentate gyrus (DG) of the hippocampus incorporates newborn neurons through adulthood. Adult-born DGCs arise from a neurogenic niche in the subgranular zone, migrate a short distance radially into the granular layer as they differentiate, and extend dendrites to form functional synapses in the molecular layer. They recapitulate the developmental steps of perinatally born DGCs and are indistinguishable from the latter after maturation. Adult newborn DGCs through adulthood [Fig. 1b]. Sparse labeling, fiducial markers on the edge of the implant and a coordinate system made it possible to find the same neuron over multiple imaging sessions (Supplementary Fig. 2). z-stacks of dendritic arbors (Fig. 1c) were traced to create digital three-dimensional reconstructions that were analyzed for different morphological parameters (Fig. 1d).

We followed the development of 33 neurons in 6 mice over time, obtaining 366 dendrite reconstructions. DGC dendrites underwent rapid growth during the third and fourth weeks after infection but then reached a near plateau with only small growth increments (Fig. 1c and Supplementary Fig. 3a). The early time points were characterized by a continuous addition and pruning of branches (Fig. 1d). There was a net increase in the number of endings during the third week after infection; however, this increase was followed by a ~28% reduction in branching during the fourth week, indicating that adult-born DGCs underwent overgrowth and dendritic pruning during their development, even though they were integrating into a pre-existing network (Fig. 1f,g). By the fifth week, the number of endings was nearly unchanged; therefore, we took the 31-dpi time point as representative of the final state of the branch structure. The maximum extent of pruning occurred at 21 dpi (Supplementary Fig. 3b), with a mean of 14.7 ± 0.54 endings per cell (s.e.m.). Sholl analysis revealed a broadening of the Sholl intersection curve over time, as a consequence of the overall arbor growth (Supplementary Fig. 3c). Most of the addition and pruning of branches took place in the 4 d around this peak (Supplementary Fig. 4a). We tracked the changes in branch structure for a subset of cells whose branches could be unambiguously matched between time points around the maximum branching. Branch addition was high on the run up to the maximum (4.0 ± 0.56 branches added per day, Supplementary Fig. 4b) but dropped sharply afterwards, whereas branch pruning tended to increase after maximum branching. The mean length of branches added between two imaging sessions was 27.8 ± 1.9 µm, which was similar to the length of pruned branches (24.3 ± 1.5 µm; Supplementary Fig. 4c). The total length added and pruned per day at these time points amounted to ~13% of the total dendrite length at the maximum of pruning (Supplementary Fig. 4d). Although most added and removed branches tended to be between 20 and 30 µm, there was considerable variability, with the occasional addition or pruning of much larger branches (Supplementary Fig. 4e).

Having established that adult-born DGCs underwent dendritic pruning during their maturation, we next investigated whether this process was modulated by experience. Adult neurogenesis is enhanced by EE and voluntary exercise, so we followed the morphological development of 30 adult-born DGC dendritic arbors (356 reconstructions, 6 mice) in mice injected with RV-GFP and exposed daily to an EE with running wheels from 7 to 60 dpi (Fig. 2a and Supplementary Fig. 5a,b). The dendrites of EE mice grew faster and were longer than those of mice raised in a regular cage (RC) at 17 to 23 dpi (Fig. 2b).
although the length of dendrites in the two groups converged by 31 dpi (Fig. 2c). Dendrites in the EE group also underwent a period of overgrowth and pruning, but the maximum branching occurred 4 d earlier than in their RC counterparts (Fig. 2d, e). EE cells extended more branches than RC cells, peaking at 17.0 ± 0.60 endings per cell (Fig. 2f), but again, this difference was no longer present by 31 dpi, as EE dendrites pruned 35% more endings than the RC group (Supplementary Fig. 5c). In fact, we could find no morphological distinction between mature DGCs in EE and RC animals, with both groups having no substantial differences in dendrite length or branch order (Supplementary Fig. 5f, g). The total number of Sholl intersections was higher in EE arbors at 17 dpi (maximum extent of branching), reflecting their faster growth at this age, but by 31 dpi the number of intersections was similar in both groups (Supplementary Fig. 5h, i). No substantial differences were found in the rate of branch addition and pruning around maximum branching or in the sizes of added and pruned branches between EE and RC neurons (Supplementary Fig. 5j–n).

We next asked whether dendrite branching depended on the extent of dendritic growth. In the hippocampus, Wnt signaling is known to be the major regulator of adult-born DGC fate and morphogenesis, with non-canonical Wnt–planar cell polarity signaling playing a strong role in the latter (see Supplementary Discussion). CELSR3, a core component of the Wnt–planar cell polarity pathway, regulates DGC dendritic growth, so we investigated how its knockdown and the consequent stunting of dendritic growth affected branching. We injected mice with a retrovirus encoding GFP and a shRNA against CELSR3 (GFP+shCELSR3) and imaged the dendritic development of 26 infected DGCs (295 reconstructions, 5 mice; Supplementary Fig. 6a). Dendrites in the GFP+shCELSR3 (knockdown) group followed a different growth pattern from their GFP counterparts: knockdown dendrites initially grew faster but their growth also subsided earlier (Fig. 2g). By 31 dpi, dendrites in the knock-
down group were shorter than in those expressing only GFP (Fig. 2h). Branching was more pronounced in the knockdown group at early time points, peaking at 17 dpi, 4 d earlier than the GFP-only group (Fig. 2i,j). However, the maximum number of endings was similar in both groups and remained similar by 31 dpi (Fig. 2k) despite the shorter length in the knockdown group, again highlighting that branch number is a relatively constant feature of mature DGCs. No difference was found in branch order or other morphological features, suggesting that DGCs can develop an adequate branching structure despite a disruption of dendritic growth (Supplementary Fig. 6b–e). Sholl analysis at 19 and 31 dpi reflected the faster growth at the former time point and the shorter dendrites at the latter (Supplementary Fig. 6f,g). No substantial differences were found in the rate of branch addition and pruning about maximum branching or in the sizes of added and pruned branches between GFP-only and GFP+shCELSR3 neurons (Supplementary Fig. 6h–l).

How do DGC dendrites mature to a similar number of branches even if they grow in mice exposed to different environments, resulting in different extents of branching during development? The answer is that not all cells undergo the same extent of pruning. On average, the fraction of endings preserved in all cells traced was 68%, but this figure depended heavily on the maximum number of endings. Cells that extended many branches pruned more than cells that extend fewer branches, both in absolute and relative terms (Fig. 3a–c). As a consequence, the coefficient of variation of the final number of endings was 32% smaller than the coefficient of variation at the maximum of branching: pruning reduced not only the number of branches but also the heterogeneity in branch numbers (Fig. 3d,e). This reduction in heterogeneity would not occur if DGCs pruned a constant absolute number or a constant fraction of branches (Supplementary Fig. 7).

Our findings therefore suggest that dendritic pruning in developing DGCs occurs in a homeostatic fashion, acting to counter excessivebranching and contributing to defining a similar branch structure for all DGCs. On the basis of our data, we hypothesize that DGCs undergo a period of net branch overgrowth that is heavily dependent on...
Pruning of DGC dendrites is homeostatic and results in reduced heterogeneity of branch numbers. (a) Plot of fraction of endings preserved and maximum number of endings with corresponding linear regression best-fit line (linear regression fit: $R^2 = 0.60, P < 0.0001$, F-test, $n = 86$ cells, 17 mice). The fraction of endings preserved depends heavily on the maximum extent of branching. (b,c) Representative examples of cells with few (b) and many (c) branches around maximum branching. (d,e) Pruning results in a reduction of branches (d; maximum branching: 16 endings; CV at maximum = 21.9%, 95% C.I. = [19.5, 25.3]; CV at 31 dpi = 14.9%, 95% C.I. = [13.0, 17.8], *$P = 0.0158$).

**Methods**

Methods and any associated references are available in the online version of the paper.

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**Author contributions**

J.T.G. and F.H.G. conceived the project, designed experiments and wrote the manuscript. J.T.G., C.W.B., S.T.J., S.T.S., S.L.P., T.T. and T.C. carried out experiments. J.T.G., C.W.B., M.S. and S.L.P. analyzed the data. J.T.G., S.T.J. and F.H.G. co-developed the DG window implant technique. F.H.G. supervised the project.

**Competing financial interests**

The authors declare no competing financial interests.

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ONLINE METHODS

Animal use. Female C57BL/6 mice (Jackson Laboratories), 6 to 7 weeks of age at the time of surgery, were used in this study. Unless otherwise noted, mice were group housed in regular cages (RC; 37.3 cm L × 23.4 cm W × 14.0 cm H, InnO Vive, San Diego, CA) under standard conditions, with up to 5 mice per cage on a 12-h light–dark cycle. Animal allocation to different experimental groups was not randomized. Animal use and procedures were approved by the Institutional Animal Care and Use Committees of the Salk Institute and the University of California San Diego. All experiments were conducted according to the US Public Health Service guidelines for animal research.

Enhanced environments. Mice assigned to EE were housed in RC from the day of surgery until 7 dpi. From that day onwards, mice were housed in groups of 5–10 in an EE cage for 12 h/d, corresponding to the dark phase of their light cycle. The large EE cage (91 cm L × 91 cm W × 30 cm H) contained a feeder, a water dispenser, a large and a small running wheel and multiple plastic tubes and domes. The various objects in the EE cage were kept constant throughout the experiment, and placement of the objects was altered only to the extent that the mice moved them within the cages. Daily exposure to the EE continued until the completion of imaging at 60 dpi. Whenever the mice were not in the EE cage, they were returned to RCs in groups of five for the duration of the light phase of their light cycle.

Viral injection. Adult newborn granule neurons were labeled with previously described retroviral vectors that expressed GFP expression under the control of a CAG (retrovirus chicken β-actin CMV) promoter: either RV-GFP3 or the previously validated RV-GFP+shCELSR3 (ref. 9) was used. These viral vectors are replication incompetent and only infect cells dividing at the time of surgery. The procedure on neuronal growth, we imaged RV-GFP–labeled newborn neurons in the right hemisphere of the hippocampus using a microinjector (Nanoject II, Drummond Science), at coordinates previously described3. Viral injection and window implantation surgery were done within 2 to 3 h of each other and the procedures on neuronal growth, we imaged RV-GFP–labeled newborn neurons were mechanically damaged or, less frequently, rendered opaque by bleeding. In certain instances, cells would extend one or more dendritic processes outside of the acquired field of view and could not be entirely reconstructed, or the field of view became too dense with processes from neighboring cells, preventing unaugible reconstruction. The full list of reconstructed dendrites is included as Supplementary Table 1. Investigators had access to experimental group allocation during data acquisition and analysis.

Statistical analysis. Every DGC analyzed had a single dendritic arbor, so the number of cells was equivalent to the number of dendritic trees. All data are presented as mean ± s.e.m. or median and interquartile range (IQR, 25th–75th percentile). Statistical comparisons were performed in Prism 6.0 (GraphPad Software) using the Mann-Whitney U-test (MWU, one independent variable, single comparison), Wilcoxon paired rank test (paired data, one independent variable), F-test, two-way ANOVA using either the Tukey or Sidak multiple comparison test (two independent variables: dpi and length per endings), or Kruskal-Wallis test using Dunn's multiple comparisons test (K-W, one independent variable: type of surgery and imaging). All statistical tests were two-tailed. Threshold for significance (α) was set at 0.05. While we did not formally test data distributions for normality, we generally chose statistical tests that do not assume a normal distribution of the data. Confidence intervals for the coefficient of variation were estimated in MATLAB using standard bootstrapping techniques (bootci.m). Sample size was not determined a priori but, after over-branching and pruning were found in the RC RV-GFP experimental group, the other experiments were designed to have a similar number of cells and mice. All graph error bars represent the s.e.m. unless otherwise specified.

Tissue collection. Mice were anesthetized with a lethal dose of ketamine and xylazine (130 mg/kg, 15 mg/kg, i.p.) and perfused transcardially with 0.9% NaCl followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Brains were dissected and postfixed in 4% PFA overnight.

Immunohistochemistry. All implanted mouse brains were collected for histological analysis. Fixed brains were transferred to a 30% sucrose solution for 24–72 h to equilibrate and were subsequently frozen and sectioned coronally at 40-μm thickness on a sliding microtome. Antibodies used were rabbit anti-Iba1 (Wako Cat. No. 019-19741) at 1:1,000 dilution, goat anti-FOS (Santa Cruz

stage via a titanium bar implant while resting on a 37 °C electrical heat blanket (Harvard Instrument). Imaging of DGCs in the dorsal leaf of the DG was done with a two-photon laser scanning microscope (MOM, Sutter Instruments) using a femtosecond-pulsed laser (Chameleon Ultra II, Coherent) tuned to 910 nm and a 40× water immersion objective (0.8 NA, Olympus). Images were acquired using the ScanImage software21, which was written in MATLAB (MathWorks). Imaging sessions started at 15 dpi and were repeated at 17, 19, 21, 22, 23, 25, 27, 29, 31, 37, 43, 48 and 60 dpi. At each time point, we acquired a three-dimensional image stack of each GFP-expressing neuron that we followed (512 × 512 pixels; 1 μm steps), taking care to include the entirety of the dendritic tree.

Analysis and quantification of dendrite morphology. Image stacks of dendritic trees were imported in TIFF format into the NeuroLucida neuron tracing software (MicroBrightField Bioscience). Cell somas and dendrites were traced manually to ensure accurate reconstruction; dendritic arbors that could not be confidently and completely reconstructed were not used in the analysis. Dendritic reconstructions were then imported into NeuroLucida Explorer (MicroBrightField Bioscience) for quantitative analysis. The dendritic parameters analyzed included the number of dendritic endings (final segments between the dendrite tip and a bifurcation), the total dendritic length (of all dendrite branches), and branch order (number of bifurcations from soma to tip of dendrites). Branch order was assigned using centrifugal numbering, and the number of branches of each order was quantified. Sholl analysis was performed by calculating the number of dendrites that intersected concentric spheres that radiated from the soma in 10-μm-radius increments. Analysis of branch addition and pruning was done with the 4DSPA software22 by visually comparing branch morphology at different time points. Data were compiled in MATLAB for further analysis. We were not able to trace all cells at all planned time points for several reasons: some cells were deemed insufficiently bright to allow the unequivocal tracing of their dendritic arbors, which happened most frequently at earlier time points; at later stages some windows were mechanically damaged or, less frequently, rendered opaque by bleeding. In certain instances, cells would extend one or more dendritic processes outside of the acquired field of view and could not be entirely reconstructed, or the field of view became too dense with processes from neighboring cells, preventing unaugible reconstruction. The full list of reconstructed dendrites is included as Supplementary Table 1. Investigators had access to experimental group allocation during data acquisition and analysis.

Fig. 8 (60x125)), indicating that the surgical implantation procedure and recurrent imaging had no effect on length and number of branches. Furthermore, we found that the density of dendritic protrusions on the ending segments of newborn neurons was similar in mice that underwent implant surgery and in non-implanted mice (Supplementary Fig. 9).

Two-photon imaging of DGC morphology. Mice were imaged under isoflurane anesthesia (1% isoflurane in O3, vol/vol) and head-fixed to the microscope
Cat. No. sc-48869) at 1:250 dilution, Cy3-conjugated donkey anti-rabbit IgG (Jackson Labs Cat. No. 711-165-152) at 1:250 dilution and Alexa 488-conjugated donkey anti-goat IgG (Jackson Labs Cat. No. 705-545-003) at 1:250 dilution. 46-Diamidino-2-phenylindole (DAPI) was used to label nuclei. Immunofluorescence images were acquired using an Olympus confocal microscope and images were processed using ImageJ.

**Fixed slice preparation and imaging.** Fixed brains were transferred to Tris-buffered saline solution (TBS; 50 mM Tris-Cl, 150 mM NaCl, pH 7.5) and sectioned coronally at 350-µm thickness on a vibratome (Leica Biosystems). Imaging was done with a two-photon laser scanning microscope (MOM, Sutter Instruments) using a femtosecond-pulsed laser (Chameleon Ultra II, Coherent) tuned to 910 nm and a 25× water immersion objective (1.05 NA, Olympus).

**Data availability.** The data that support the findings of this study, including all dendritic tree reconstructions, are available from the corresponding author upon request.

A **Supplementary Methods Checklist** is available.

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