

# Propofol Anesthesia Impairs the Maturation and Survival of Adult-born Hippocampal Neurons

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## ABSTRACT

**Background:** Adult neurogenesis occurs in the hippocampus of most mammals, including humans, and plays an important role in hippocampal-dependent learning. This process is highly regulated by neuronal activity and might therefore be vulnerable to anesthesia. In this article, the authors investigated this possibility by evaluating the impact of propofol anesthesia on mouse hippocampal neurons generated during adulthood, at two functionally distinct maturational stages of their development.

**Methods:** Adult-born hippocampal neurons were identified using the cell proliferation marker bromodeoxyuridine or a retroviral vector expressing the green fluorescent protein in dividing cells and their progenies. Eleven or 17 days after the labeling procedure, animals ( $n = 3-5$  animals per group) underwent a 6-h-long propofol anesthesia. Twenty-one days after labeling, the authors analyzed the survival, differentiation, and morphologic maturation of adult-born neurons using confocal microscopy.

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Received from the Department of Fundamental Neurosciences, University of Lausanne, Switzerland. Submitted for publication June 29, 2012. Accepted for publication November 14, 2012. This work was supported by the Swiss National Science Foundation (Bern, Switzerland) grant PP0033-119026 (N.T., M.K., S.S., and J.S.), the Novartis foundation, Basel, Switzerland (M.K.), the Swiss National Science Foundation Grant 31003A-130625 (L.V.), and by the CRUS-SCIEX grant 10-050 (L.V. and K.D.). Images acquisition was performed at the Cellular Imaging Facility of the University of Lausanne, Lausanne, Switzerland.

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## What We Already Know about This Topic

- General anesthetics have powerful effect on neuronal development and differentiation in young animals, but their effects on adult neurogenesis are less certain
- Neurogenesis in the adult hippocampus plays an important role in learning and memory and might be sensitive to anesthesia exposure

## What This Article Tells Us That Is New

- Adult-born mouse hippocampal neurons are vulnerable to propofol anesthesia, which reduced neuron survival and dendritic maturation *in vivo*
- Effects on hippocampal neurogenesis could potentially result in neurocognitive deficits after anesthesia

**Results:** Propofol impaired the survival and maturation of adult-born neurons in an age-dependent manner. Anesthesia induced a significant decrease in the survival of neurons that were 17 days old at the time of anesthesia, but not of neurons that were 11 days old. Similarly, propofol anesthesia significantly reduced the dendritic maturation of neurons generated 17 days before anesthesia, without interfering with the maturation of neurons generated 11 days before anesthesia.

**Conclusions:** These results reveal that propofol impairs the survival and maturation of adult-born hippocampal neurons in a developmental stage-dependent manner in mice.

**A**DULT neurogenesis results in the continuous generation of new neurons (referred to as adult-born neurons) which integrate into the brain circuitry throughout adulthood.<sup>1</sup> In most mammalian species, including humans,<sup>2</sup> adult neurogenesis occurs in two brain areas: the hippocampus and the olfactory bulb. In the hippocampus, adult neurogenesis plays a role in cognition: the experimental reduction of adult neurogenesis in rodents results in learning and memory impairment,<sup>3-5</sup> and inversely, learning tasks and antidepressants increase adult neurogenesis,<sup>6,7</sup> which in turn increase learning performances.<sup>8</sup> Adult neurogenesis consists of five discrete processes, which are regulated independently: (1) proliferation of the stem cells, (2) differentiation of the progenies into neuronal lineage, (3) migration of the new cells into the granule cell layer, (4) survival of the new cells, and finally (5) maturation/integration of the new cells into the existing neuronal network.<sup>9</sup> Interestingly, neuronal activity plays a strong regulatory role on each of these steps of

adult neurogenesis,<sup>9–12</sup> thereby enabling a bidirectional regulation of neuronal activity and adult neurogenesis.

During the development of the central nervous system, neuronal maturation is particularly sensitive to external stimuli.<sup>13</sup> General anesthetics, through their interactions with ligand-gated ion channels, are powerful modulators of neuronal activity and can potentially interfere with neuronal development. In line, extensive experimental data suggest that, at distinct stages of development, these drugs can induce neuroapoptosis or impair neuronal differentiation.<sup>14,15</sup> Adult-born neurons also undergo intense, activity-dependent development and thus may be particularly sensitive to anesthesia exposure. In this study, we tested this vulnerability by examining the effect of propofol anesthesia on the development of neurons generated in the mature mouse hippocampus. In particular, we investigated how propofol impacts on the maturation and survival of new neurons and whether this effect might depend on the developmental stage of these cells. To this aim, we labeled neuronal progenitors in the dentate gyrus of the mouse hippocampus *in vivo* using both the cell proliferation tracer bromodeoxyuridine (BrdU) and viral-mediated gene delivery.

To evaluate the impact of propofol exposure on the development of these cells, adult mice were anesthetized for 6 h at two different developmental time points: 11 or 17 days after BrdU or viral injection (days postinjection, dpi). The impact of this anesthesia protocol on the survival and morphology of these cells was examined using confocal microscopy at 21 days after BrdU or viral injection, a time-point by which these adult-born neurons functionally integrate into the hippocampal network.<sup>16–18</sup>

## Material and Methods

### Experimental Animals

The animals used for this study were 8- to 10-week-old C57BL6/J male mice. They were purchased from Janvier (Le Genest Saint Isle, France) and were group-housed in standard cages under light- (12 h light/dark cycle) and temperature-controlled (22°C) conditions. Food and water were available *ad libitum*. Every effort was made to minimize the number of animals used and their suffering, and experimental protocols were approved by the Swiss animal experimentation authorities (Service de la consommation et des affaires vétérinaires, Epalinges, Switzerland).

### Anesthesia Procedure

Animals were anesthetized for 6 h, starting by an initial injection of propofol (intraperitoneal, 100 mg/kg body weight, propofol 1% medium-chain triglycerides; Fresenius Kabi, Oberdorf, Switzerland) followed by five subsequent injections at 50 mg/kg at a rate of one injection per hour. This regimen induced deep sedation, as verified by the absence of the righting reflex. The effect of this anesthesia protocol on cardiorespiratory function was examined on venous blood

gases values obtained through right atrial puncture using the handheld i-STAT analyzer (Abbott point of care Inc., Birmingham, United Kingdom) and the Bayer Ascensia Contour (Bayer Health Care, Tarrytown, NY) blood glucose device, and it showed no modification of pH, pCO<sub>2</sub>, pO<sub>2</sub>, glucose level, and hemoglobin level (table 1). Control mice were injected with the same regimen and volume of vehicle: six injections of soybean oil (Lipofundin medium-chain triglycerides/long-chain triglycerides 20%; Braun, Sempach, Switzerland). During anesthesia, animals were kept on a homeothermic blanket system with a temperature probe under the body of one animal to maintain body temperature at 37°C (Harvard Apparatus, Holliston, MA).

### BrdU Administration and Immunohistochemistry

Twenty-four mice were injected intraperitoneally with 50 mg/kg of BrdU (Sigma-Aldrich, Buchs, Switzerland) twice, at 2-h interval. BrdU intercalates into the nascent DNA strand during cell division and therefore labels dividing cells. Eleven or 17 days later, mice were either anesthetized for 6 h (n = 5 per time-point) or administered three vehicle injections (n = 4 mice per group) or one vehicle injection (n = 3 mice per group). All animals were then perfusion-fixed 21 days after the BrdU injections (fig. 1A). Brains were then dissected, cryoprotected and 40-μm thick sections were cut on a cryomicrotome, and treated for immunohistochemistry as previously described.<sup>19</sup> In brief, slices were first incubated in 50% formamide/50% 2× saline sodium citrate buffer (2× saline sodium citrate is 0.3 M NaCl and 0.03 M sodium citrate, pH 7.0) at 65°C for 2 h, rinsed twice in 2X saline sodium citrate buffer, incubated in 2 M HCl for 30 min at 37°C, and rinsed in 0.1 M borate buffer pH 8.5 for 10 min. Sections were then blocked with 3% normal goat serum (Invitrogen, Basel, Switzerland) and Triton 0.25% (Sigma-Aldrich) in phosphate buffer saline. Primary and secondary antibodies were diluted in 0.1 M phosphate buffer saline containing 0.125% Triton and 3% normal goat serum. The following primary antibodies were used: mouse anti-BrdU (Chemicon International, Dietikon, Switzerland; 1:250) 72 h at 4°C and Alexa 594 (Invitrogen; 1:250) goat anti-mouse.

To examine the neuronal differentiation of the BrdU-positive cells, we performed a double-labeling using rat anti-BrdU (Abcam, Cambridge, United Kingdom; 1:250) and mouse anti-Neu-N (Chemicon International; 1:500), combined with the goat anti-mouse secondary antibodies Alexa 594 (Invitrogen; 1:250) and Alexa 488 (Invitrogen; 1:250), respectively.

All cell counts were conducted blind with regards to the mouse status and using stereological principles: one section out of six was immunostained (eight to nine sections per animal), and all BrdU-immunoreactive cells were counted in the entire thickness of the sections. Depending on the condition, we found an average of 15–30 cells/section (for a total of more than 150 cells per animal). The number of cells/section was then averaged for each mouse. To assess the volume of the dentate gyrus, we measured the surface

**Table 1.** Blood Physiologic Parameters Remain within Normal Range during Anesthesia

	Control	Propofol
pH	7.35 ± 0.04	7.35 ± 0.04
pCO <sub>2</sub> (kPa)	6.76 ± 0.4	6.55 ± 0.6
Glucose (mg dl <sup>-1</sup> )	9.75 ± 0.6	9.48 ± 1
Hemoglobin (g dl <sup>-1</sup> )	10.1 ± 0.6	9.8 ± 0.6

After 6 h of propofol anesthesia or vehicle injection, blood parameters were similar between groups ( $P > 0.1$ , unpaired two-tailed Student  $t$  test,  $n = 4$ ). Data are expressed as mean values ± SEM.

of the granule cell layer and multiplied it by the thickness of the sections. For colocalization of BrdU and Neu-N, 20–40 BrdU-immunoreactive cells per animal were examined using confocal microscopy and colocalization of both markers was confirmed on single optical sections. The proportion of double-immunopositive (BrdU and Neu-N) was obtained for each animal and then averaged for each group.

### Retrovirus-mediated Labeling

We used a retroviral vector derived from the Moloney murine leukemia virus containing a green fluorescent protein expression cassette under the control of the cytomegalovirus early enhancer and chicken beta-actin promoter (cag), as previously described.<sup>17,20</sup> The concentrated viral solution was prepared using Human Embryonic Kidney 293T cells as a package cell line and collected through ultraspeed centrifugation. Further purification was performed using acrodisc units with Mustang Q membrane (VWR International AG, Schlieren, Switzerland). The final virus titer was  $10^8$  pfu/ml, as measured by green fluorescent protein-expressing colony formation on 293T cells. Mice were anesthetized with isoflurane using an anesthetic mask (Narishige, London, United Kingdom), and 1  $\mu$ l of virus was injected into the dentate gyrus of either one or two hippocampi of the mouse (stereotaxic coordinates from Bregma: –2 mm anteroposterior; –1.75 mm lateral; –2.25 mm ventral).

Brain sections of one-in-two series were selected for analysis. Green fluorescent protein signal was amplified by immunohistochemistry using chicken anti-green fluorescent protein IgG (Aves Labs, Tigard, OR; 1:1000) and DyLight 488 goat anti-chicken IgY (Jackson ImmunoResearch Europe Ltd., Suffolk, United Kingdom; 1:250). 4,6 Diamidino-2-phenylindole was used to reveal nuclei. One of the mice anesthetized at 11 dpi did not show any labeled neurons (the injection site was out of the dentate gyrus). We therefore could not analyze it.

### DiI Labeling of Dendrites

The mice were perfused as described before.<sup>19</sup> Immediately after perfusion, 50- $\mu$ m thick vibratome sections were stained

by placing small crystals of a DiI cell-labeling solution (Invitrogen) in the inner molecular layer of the dentate gyrus. Labeled dendrites were imaged shortly thereafter.

### Iontophoretic Intracellular Injections

Granule cells from the most external part of the granule layer of the dentate gyrus were injected iontophoretically with a Lucifer yellow solution and immunostained against Lucifer yellow on coronal mouse brain sections as described previously.<sup>21</sup>

### Confocal Microscopy and Analysis

Hippocampal sections were imaged using a Leica SP5 AOBs laser scanning microscope (Chalmers University of Technology, Gothenburg, Sweden). Global views of green fluorescent protein-labeled adult-born neurons were imaged with a 40 $\times$  oil lens and a z-step of 5  $\mu$ m, and dendrites were imaged with a 63 $\times$  oil lens and a z-step of 0.38  $\mu$ m. Two-dimensional maximum intensity projections of each z-series were created with Fiji software.<sup>#</sup> Dendritic length and spine density were measured with Fiji and Trace software.<sup>\*\*</sup> The perimeter and the area of the mossy fiber terminals were determined using Fiji software on maximal intensity projections. The circularity of the terminals was calculated with the following formula: circularity =  $4 \times \pi \times \text{area}/(\text{perimeter})^2$ .

### Statistical Analysis

The following morphologic parameter estimations were compared between control and anesthesia groups (11 dpi and 17 dpi): number of immunoreactive cells, maximal extent of their dendritic arbor, dendritic spine density, projection area, circularity, and number of filopodia per bouton.

Hypothesis testing was two-tailed. All analysis were performed using R 2.15.1 software (R foundation for statistical computing, Institute for Statistics and Mathematics, Vienna, Austria) and GraphPad Prism 6 (Graphpad software Inc., La Jolla, CA). First, Shapiro-Wilk tests were performed on each group of data to test for distribution normality. When the distribution was not normal, nonparametric Kruskal-Wallis test was applied. Otherwise, the analysis was performed using parametric tests (one-way ANOVA followed by a *post hoc* unpaired  $t$  test and a Bonferroni correction). For two-sample comparisons, the equality of variances of the groups was tested and the adequate unpaired  $t$  test was used. The frequency distributions of the dendritic extensions (bins from 30 to 100% dendritic extension) were compared using contingency tables and a Mantel-Haenszel chi-square test ( $\chi^2$ ). Data are presented as mean ± SEM.

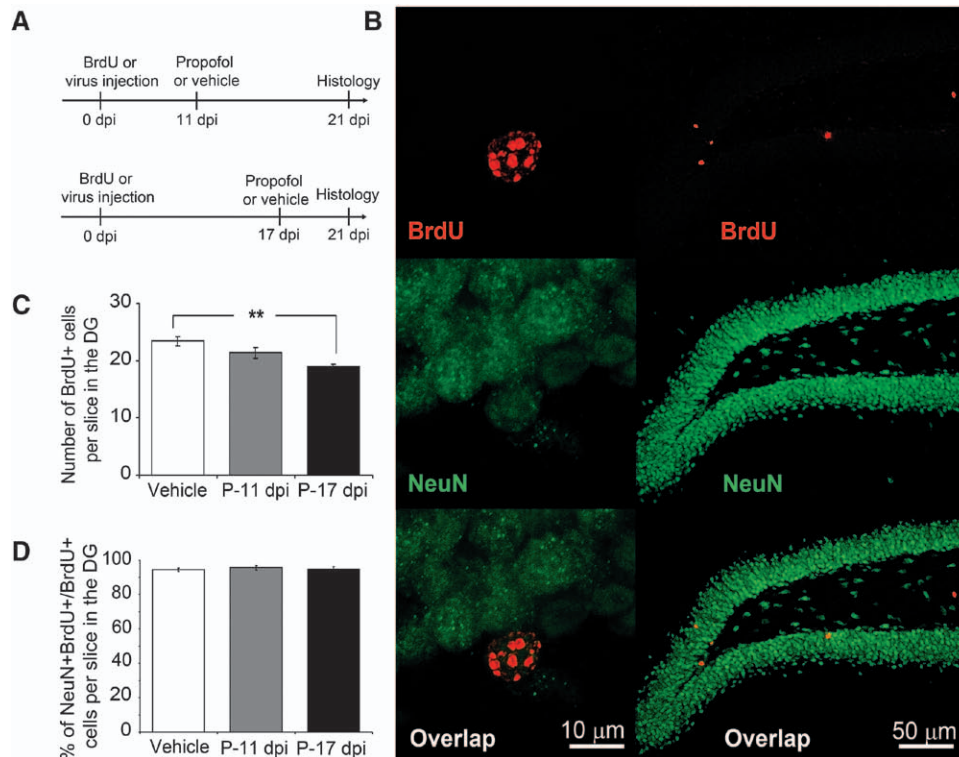
## Results

To measure the effect of propofol anesthesia on new cell survival and differentiation, we labeled dividing cells with BrdU and identified the newly formed neurons with

<sup>#</sup> Freely available at <http://fiji.sc/>. Accessed November 20, 2012.

<sup>\*\*</sup> Freely available at <http://synapses.clm.utexas.edu/tools/trace/trace.stm>. Accessed November 20, 2012.





**Fig. 1.** Anesthesia decreases the survival of adult-born neurons. (A). Experimental timeline: All mice were injected with either BrdU or retrovirus at day 0 (0 dpi). Propofol or vehicle was then injected 11 or 17 days later, followed by perfusion and histology at 21 dpi. (B). Confocal micrographs of the dentate gyrus stained for NeuN (green) and BrdU (red). All values are presented as mean  $\pm$  SEM.  $**P < 0.01$ . (C). Histogram showing the number of BrdU-immunostained cells per slice. ANOVA between groups  $F(2,21) = 6.14$ ,  $P = 0.0079$ , Bonferroni *post hoc* test between groups  $P < 0.01$ . (D). Proportion of BrdU-stained cells that expressed the neuronal marker Neu-N. ANOVA between groups  $F(2,21) = 0.21$ ,  $P = 0.81$ . BrdU = bromodeoxyuridine.

immunostaining for the neuron-specific marker Neu-N. A total of 24 mice were administered two doses of 50 mg/kg of BrdU at 2-h interval and were anesthetized for 6 h at either 11 ( $n = 5$ ) or 17 ( $n = 5$ ) days after BrdU injection (days postinjection, dpi). This experimental design therefore enabled us to test the effect of anesthesia on adult-born neurons at two different maturation stages. Control mice were intraperitoneally injected with the equivalent regimen of vehicle at the same time points: four mice received three injections of vehicle and three mice received a single injection of vehicle. In a separate experiment aimed at examining the effect of this anesthesia paradigm on cardiorespiratory function, we found that 6 h propofol anesthesia did not modify pH,  $pCO_2$ ,  $pO_2$ , glucose level, and hemoglobin level (table 1). Twenty-one days after BrdU injection, all mice were perfused, and one of six hippocampal sections was immunostained for BrdU and Neu-N. Using Neu-N immunostaining to determine the contour of the granule cell layer, we found that the volume of the granule cell layer was unchanged between groups (ANOVA  $F(2,21) = 0.6$ ,  $P = 0.55$ ). All BrdU-immunoreactive cells were counted in the entire thickness of the sections to yield an average of cells/slice for each animal (fig. 1A). The number of BrdU-immunoreactive cells was significantly reduced in the group anesthetized at 17 dpi compared with the control group (17 dpi:  $18.86 \pm 0.57$ ; Ctrl:  $23.38 \pm 0.77$ ;

multiple-comparison ANOVA test:  $F_{2,21} = 6.14$ ,  $P = 0.008$ , Bonferroni correction  $P < 0.01$ ; fig. 1B), indicating that propofol anesthesia at this developmental time-point decreased the survival of adult-born cells. Coexpression of BrdU and Neu-N was then analyzed on single confocal planes. In all experimental groups, the proportion of BrdU-immunoreactive cells that coexpressed Neu-N was similar between conditions (Ctrl:  $94.2 \pm 1$ ; 11dpi:  $95.4 \pm 1.4$ ; 17dpi:  $94.7 \pm 1.6$ ; one-way ANOVA:  $F_{2,21} = 0.21$ ;  $P = 0.8$ ; fig. 1, C and D), indicating that propofol anesthesia did not interfere with neuronal differentiation. The results of the four control groups were comparable, and the corresponding data were subsequently pooled. Thus, propofol anesthesia reduced the survival, but not the differentiation of neurons generated 17 days before anesthesia, and did not affect the differentiation or survival of neurons generated 11 days before anesthesia.

To test whether propofol anesthesia also interfered with the maturation of adult-born neurons, we examined their dendritic arbor. We identified adult-born neurons with retroviral-mediated green fluorescent protein expression, thereby enabling the visualization of the whole architecture of adult-born neurons<sup>20,22</sup> (fig. 2A). For each neuron (90–200 neurons per mouse, three to four mice per group), we measured the maximal extent of its dendritic arbor as a percentage of the full extent of the molecular layer at 21

dpi (fig. 2B). Adult-born neuron dendritic extensions were classified in bins and the frequency distributions compared using a contingency table. We found that propofol anesthesia at 17 dpi, but not at 11 dpi, significantly reduced the relative proportion of adult-born neurons with long dendritic trees in favor of neurons with short dendritic trees. Comparisons were made between groups using the frequency distributions of each group (control *vs.* 17 dpi:  $\chi^2_{21} = 39.7$ ,  $P < 0.001$ ; control *vs.* 11 dpi:  $\chi^2_{21} = 1.21$ ;  $P = 0.27$ ; fig. 2C) and then the frequency distribution of the means per animal of each group (control *vs.* 17 dpi:  $\chi^2_{21} = 39.7$ , degrees of freedom = 1,  $P < 0.001$ ; control *vs.* 11 dpi:  $\chi^2_{21} = 1.21$ ,  $P = 0.27$ ; fig. 2C). To examine whether the effect of propofol was restricted to adult-born neurons, we also analyzed the morphology of control, prenatally generated neurons, which form the external third of the granule cell layer.<sup>23</sup> These cells were iontophoretically injected with the fluorescent dye Lucifer yellow to enable their identification and the analysis of their dendritic extension (13–19 neurons per mouse, four to five mice per group). We observed no morphologic difference on mature neurons between control and anesthetized groups because virtually all neurons displayed dendrites that extended to the tip of the molecular layer (fig. 2C). Thus, propofol reduced the dendritic maturation of neurons generated 17 days before anesthesia, without affecting the morphology of neurons generated 11 days before anesthesia or the morphology of neurons generated prenatally.

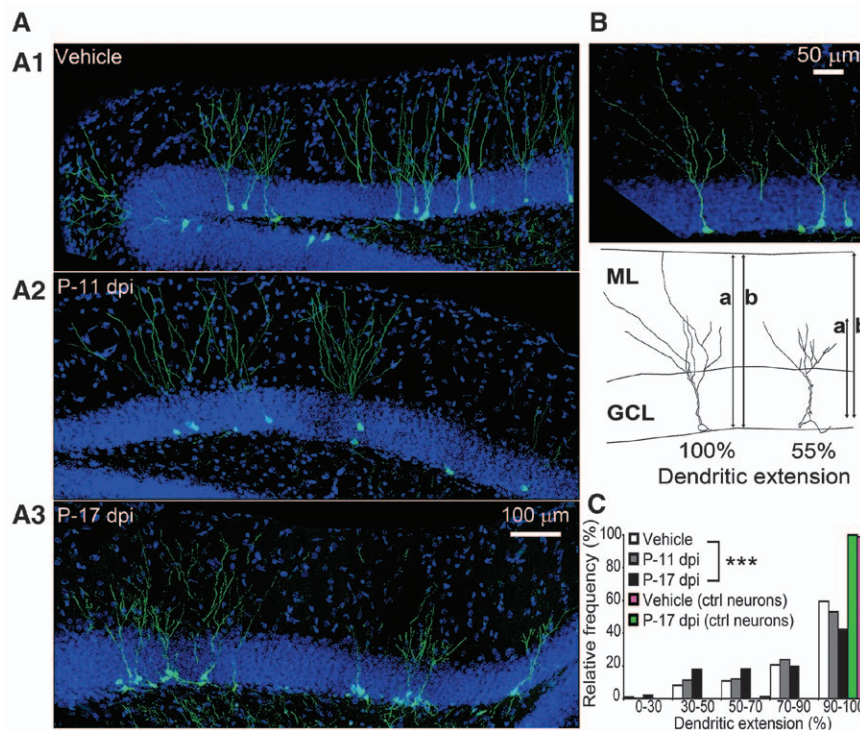
Finally, to estimate the synaptic integration of newly born hippocampal neurons, we examined their dendritic spine density in the inner third (composed mainly of hilar/commissural afferences) and in the middle third (composed mainly of entorhinal cortex afferences) of the molecular layer and the morphology of their mossy fiber boutons in the CA3 area (consisting of their efferences on pyramidal neurons; fig. 3). We found that propofol did not alter spine density on adult-born neurons in both types of inputs (spine density in the inner third of the molecular layer:  $0.49 \pm 0.06$  spines/ $\mu\text{m}$  in control animals *vs.*  $0.42 \pm 0.08$  spines/ $\mu\text{m}$  in animals anesthetized at 17 days; spine density in the middle third of the molecular layer:  $0.71 \pm 0.06$  spines/ $\mu\text{m}$  in control animals *vs.*  $0.79 \pm 0.11$  spines/ $\mu\text{m}$  in animals anesthetized at 17 days. 900–3000 spines, 24–78 neurons, and three to four mice per condition; Kruskal-Wallis test,  $P = 0.44$  and  $P = 0.33$ , respectively; fig. 3A). We examined the spine density on control neurons of the external granule cell layer that were microinjected with Lucifer yellow, and we also labeled random dendritic branches on each mouse using the lipophilic dye DiI. In both of these populations of neurons, we did not detect any effect of anesthesia on spine density (460–2450 spines, 19–27 neurons, three to five mice per condition; spine density in the inner third of the molecular layer:  $1.66 \pm 0.22$  spines/ $\mu\text{m}$  in control animals *vs.*  $1.58 \pm 0.13$  spines/ $\mu\text{m}$  in animals anesthetized at 17 days; spine density in the middle third of the molecular layer:  $1.51 \pm 0.19$  spines/ $\mu\text{m}$  in control animals

*vs.*  $1.40 \pm 0.12$  spines/ $\mu\text{m}$  in animals anesthetized at 17 days; spine density of control dendrites in the middle third of the molecular layer:  $1.76 \pm 0.03$  spines/ $\mu\text{m}$  in control animals *vs.*  $1.54 \pm 0.13$  spines/ $\mu\text{m}$  in animals anesthetized at 17 days; unpaired *t* test,  $P = 0.74$ ,  $P = 0.64$ , and  $P = 0.28$ , respectively; fig. 3B). Similarly, we found no effect of anesthesia on the morphology of mossy fiber boutons when we analyzed their projection area, circularity, and the number of filopodia per bouton (three to four mice per condition; 11–21 boutons per mouse; area:  $5.12 \pm 0.6 \mu\text{m}^2$  in control animals *vs.*  $5.25 \pm 0.6 \mu\text{m}^2$  in animals anesthetized at 11 dpi and  $4.8 \pm 0.2 \mu\text{m}^2$  in animals anesthetized at 17 dpi, Kruskal-Wallis test:  $P = 0.70$ ; circularity:  $0.49 \pm 0.03$  in control animals *vs.*  $0.49 \pm 0.04 \mu\text{m}^2$  in animals anesthetized at 11 dpi and  $0.56 \pm 0.03$  in animals anesthetized at 17 dpi, one-way ANOVA:  $P = 0.27$ ; number of extensions per bouton:  $2.7 \pm 0.47$  in control animals *vs.*  $2.49 \pm 0.3 \mu\text{m}^2$  in animals anesthetized at 11 dpi and  $2.6 \pm 0.29$  in animals anesthetized at 17 dpi, Kruskal-Wallis test:  $P = 0.97$ ; fig. 3, D–H). Thus, propofol anesthesia did not alter the formation of input and output synapses on hippocampal granule neurons generated prenatally or during adulthood.

## Discussion

In this study, we found that propofol anesthesia significantly impaired the survival and dendritic maturation of adult-born hippocampal neurons. Pulse-chase experiments using the cell proliferation marker BrdU in combination with neuron-specific immunomarkers demonstrated that these effects depend on the age of the immature neurons at the time of anesthesia because propofol exposure led to a significant decrease in the survival of neurons that were 17 days old but not 11 days old at the time of anesthesia. In line with these observations, retroviral labeling and follow-up of dentate gyrus progenitor cells revealed that propofol severely impaired dendritic arbor extension of 17-day-old neurons, whereas it had no effect on 11-day-old cells. Altogether, these results point to a developmental stage-dependent impact of propofol anesthesia on differentiating adult-born hippocampal neurons. Because the appropriate maturation and the related integration of adult-born hippocampal neurons into the hippocampal circuitry play a pivotal role in cognitive processing, our study suggests a potential framework to explain the cellular mechanisms through which anesthetics impair cognitive function in adults.

The aim of this study was to examine the possibility that anesthesia may interfere with hippocampal adult neurogenesis. Increasing experimental evidence indicates that adult-born neurons play an important role in information processing underlying spatial cognition and learning.<sup>24–26</sup> Moreover, it is now well established that  $\gamma$ -aminobutyric acid (GABA)-mediated neuronal activity plays a pivotal role in the integration of newly generated hippocampal neurons into the existing circuitry.<sup>11,12,27–30</sup> In this context, because the majority of general anesthetics are powerful potentiators of neurotransmission *via*



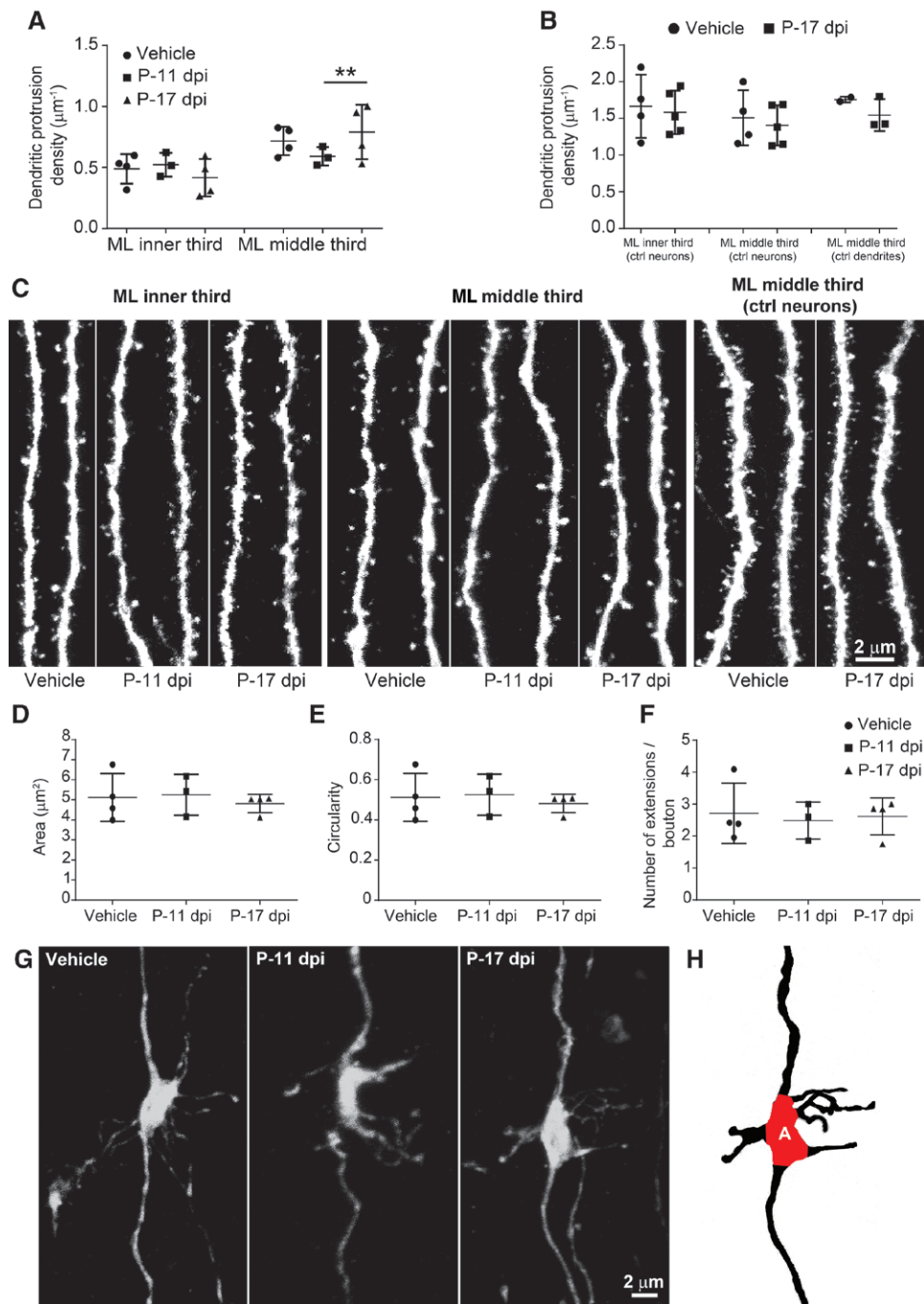
**Fig. 2.** Anesthesia reduces the dendritic extension of adult-born neurons. (A). Confocal micrographs of the dentate gyrus of vehicle-treated animals (A1), animals anesthetized at 11 dpi (A2), and animals anesthetized at 17 dpi (A3). (B). Schematic illustration of the morphometric analyses: dendritic extension =  $a/b \times 100$  ( $a$  is the distance between the center of the cell body and the tip of the longest dendrite;  $b$  is the distance between the center of the cell body and the end of the molecular layer). (C). Histogram showing the relative frequency of neurons per dendritic extension range. Chi-square test; control versus 17 dpi:  $P < 0.001$  (\*\*\*) ; control versus 11 dpi:  $P = 0.27$ ,  $n = 3-4$ . ML = molecular layer; GCL = granule cell layer.

GABA A receptors,<sup>31</sup> exposure to these drugs could impede the physiologic neural activity patterns required for the maturation and integration of adult-born neurons. To elucidate this hypothesis, we focused on propofol because this drug is a powerful potentiator of GABAergic neurotransmission and is widely used in clinical practice. Further studies will determine whether other general anesthetics exert similar effects on adult hippocampal neurogenesis.

We found that exposure to propofol significantly decreased the survival of adult-born neurons in a developmental stage-dependent manner. These results are seemingly contradictory to recent observations indicating that the general anesthetic isoflurane does not affect the survival of adult-born neurons in the hippocampus of aged rats.<sup>32</sup> Although we cannot formally exclude that these two anesthetics differentially affect hippocampal neurogenesis, we believe that targeting different stages of neurogenesis might better explain the discrepancies between these studies. Indeed, by exposing animals to anesthesia and immediately thereafter evaluating the impact of this treatment on neurogenesis using BrdU pulse-chase paradigms, Stratmann *et al.*<sup>32</sup> investigated how anesthesia interferes with neuronal proliferation, differentiation, and survival. In our study, in contrast, we labeled adult-born neurons several days before anesthesia and examined the effect of general anesthesia on already differentiated adult-born

neurons. This strategy is fundamentally different as it is based on pulse-labeling a population of proliferating progenitors. It enables us to track the differentiation of this labeled population over time and evaluate the effects of propofol on the differentiation and survival of these cells at distinct maturational stages. Results using this approach revealed a differential effect of propofol on 17-day-old neurons compared with 11-day-old neurons, where propofol reduced the maturation and survival rate of 17-day-old neurons but not of 11-day-old neurons. Several mechanisms might account for these effects. This developmental stage-dependent susceptibility to propofol may result from the expression of the KCC2 chloride transporters, which occurs at around 14 days after cell division in adult-born neurons.<sup>12</sup> The expression of KCC2 transporters results in GABA receptor activity to switch from depolarizing to hyperpolarizing and thus to invert the effect of GABA or its agonists on neurons.<sup>33</sup> Interestingly, knocking down the NKCC1 transporter, which results in a premature hyperpolarizing effect of GABA, induces a maturation delay of adult-born neurons similar to the effect observed in our study.<sup>12</sup> This supports the view that GABA receptor activity is required for the proper maturation of adult-born neurons<sup>11,27-30,34</sup> and that the exposure of immature neurons to propofol may induce maturation defects. However, the maturation of adult-born neurons involves several other





**Fig. 3.** Anesthesia has no effect on spine density and mossy fiber terminals of adult-born neurons. (A). Scatter plot showing dendritic spine density in the inner and middle thirds of the molecular layer in adult-born neurons. One-way ANOVA: inner third:  $F = 1.37$ ,  $P = 0.31$ ; middle third:  $F = 0.64$ ,  $P = 0.55$ ,  $n = 3-4$ . (B). Scatter plot of the dendritic spine density in the inner and middle thirds of the molecular layer in control neurons, that is, granule neurons from the outer third of the granule cell layer, or random dendrites labeled with Dil. Unpaired two-tailed Student  $t$  test:  $P > 0.1$ ,  $n = 2-5$ . Data are expressed as mean values  $\pm$  SEM. (C). Confocal micrographs of dendritic segments for vehicle-injected mice (Vehicle), mice anesthetized at 11 dpi (P-11 dpi), and mice anesthetized at 17 dpi (P-17 dpi). (D). Scatter plot showing mossy terminal projection area. One-way ANOVA:  $F = 0.21$ ,  $P = 0.81$ . (E). Scatter plot of the circularity of mossy fiber terminals. One-way ANOVA:  $F = 1.5$ ,  $P = 0.27$ . (F). Scatter plot showing the number of filopodial extensions per mossy fiber terminal. One-way ANOVA:  $F = 0.08$ ,  $P = 0.9$ . (G). Confocal micrographs of mossy fiber terminals representative for each condition. (H). Schematic illustration of the perimeter and projection area (A) measurement for a mossy fiber terminal. dpi = days postinjection; ML = molecular layer.

developmental steps, such as changes in *N*-methyl-D-aspartic acid receptor composition<sup>35</sup> and of synaptic connectivity.<sup>36</sup> Therefore, the differential effect of anesthesia at 11 dpi and 17 dpi may be due to several factors, which might act alone or in combination.

The most likely explanation to the smaller dendritic extension observed in the P-17 dpi group after propofol treatment is that anesthesia impairs the development of adult-born neurons. This conclusion is in line with our previous observation of the effect of propofol during postnatal development<sup>37</sup> and the observation that GABA receptor activity in adult-born neurons is necessary for the development of dendritic tree.<sup>12</sup> An alternative explanation is that propofol anesthesia induces the selective loss of neurons with larger dendritic extensions in the P-17 dpi group. Further experiments aimed at examining neuronal death under propofol exposure may contribute to tease out these possibilities.

Our observations that propofol anesthesia did not modify dendritic spine density on adult-born granule neurons are different from our previous studies, in which we found that anesthesia affects spine density on pyramidal neurons in the early postnatal cortex and hippocampus.<sup>21,37,38</sup> This discrepancy suggests that the effect of propofol on dendritic spine density is specific to postnatal development and supports the view that it may be mediated by network activity rather than being entirely cell autonomous. It is, nevertheless, important to note that the propofol-induced reduction in dendritic length on adult-born neurons, as observed in the current study, reduces the overall axospinous innervation on new neurons. In a previous experiment, we showed that inhibiting neuronal activity specifically on adult-born neurons results in a decrease in their maturation and survival, whereas inhibiting neuronal activity of the whole hippocampal network results in an increase of the maturation and survival of adult-born neurons.<sup>10</sup> Thus, both network and cell autonomous activity may play a critical role for the survival and maturation of adult-born neurons and a pharmacologic treatment such as anesthesia likely results in a mixed effect.

Further experiments will be necessary to decipher the mechanisms by which propofol reduces the survival and maturation of this population of adult-born neurons and to examine whether this effect is cell autonomous or is mediated by an effect on network activity.

The physiological relevance of our findings and the persistence of the maturation deficit remain to be determined. Nevertheless, our results indicate that a single event of propofol anesthesia can reduce the maturation and survival of neurons that are at a critical developmental phase during the procedure. This effect may in turn induce learning deficits. Indeed, ablation or stimulation studies indicate that immature adult-born neurons play a significant role in hippocampal function<sup>3,24,39,40</sup> and in learning.<sup>8,41</sup> Interestingly, recent results indicate that ablating neurogenesis in a specific time window results in long-lasting learning impairment.<sup>3,39–42</sup> Furthermore, learning induces structural plasticity of adult-born neurons, which in turn contribute to the network remodeling induced

by learning.<sup>26</sup> Thus, by reducing the survival and modifying the dendritic tree structure of adult-born neurons, anesthesia may interfere with the plastic properties of these cells and contribute to anesthesia-induced, postoperative memory impairment and cognitive deficit. In line with this possibility, previous rodent works report anesthesia exposure-related hippocampal memory impairment.<sup>43–45</sup> In these experiments, iso-flurane/nitrous oxide anesthesia impaired acquisition of new tasks either in the young or in the aged rats, whereas retrieval of consolidated memory was only reduced in aged animals. More recent data, however, revealed no effect of a 2-h-long propofol exposure on spatial working memory as tested by the 12-arm radial maze test.<sup>46</sup> Future work is needed to determine the exposure time-dependent impact of propofol on this and other, more subtle, memory tasks.

In conclusion, this study demonstrates that propofol anesthesia impairs the survival and maturation of adult-born hippocampal neurons in mice. These results are in line with previous series of observations demonstrating the importance of GABAergic neural activity in the development of these cells.<sup>12</sup> Given the important role of adult hippocampal neurogenesis in the lifelong acquisition of new memories, our findings might also provide potential mechanisms underlying impaired cognitive function in the postoperative period.

The authors thank Rudolf Kraftsik, B.S. (Senior Scientist, Department of Fundamental Neurosciences, University of Lausanne, Lausanne, Switzerland), for statistical and image analysis.

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