Title: Propofol anesthesia impairs the maturation and survival of adult-born hippocampal neurons.
Authors: Krzisch M, Sultan S, Sandell J, Demeter K, Vutskits L, Toni N
Journal: Anesthesiology
Year: 2013 Mar
Volume: 118
Issue: 3
Pages: 602-10
DOI: 10.1097/ALN.0b013e3182815948

Anesthesiology
Perioperative Medicine Investigations
Animal Study

Propofol anesthesia impairs the maturation and survival of adult-born hippocampal neurons

Marine Krzisch, M.Sc§; Sébastien Sultan, PhD#; Julie Sandell, M.Sc§, Kornél Demeter, PhD∫; Laszlo Vutskits, M.D. PhD π , Nicolas Toni, PhD*
§ PhD Student, Department of Fundamental Neurosciences, University of Lausanne, Switzerland
# Postdoctoral fellow, Department of Fundamental Neurosciences, University of Lausanne, Switzerland
∫ Research assistant, Department of Fundamental Neuroscience, University of Geneva Medical School, Switzerland and Laboratory of Cellular and Developmental Neurobiology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary
π Senior Lecturer, Department of Anesthesiology, Pharmacology and Intensive Care, University Hospital of Geneva; Department of Fundamental Neuroscience, University of Geneva Medical School, Switzerland

* Assistant Professor, Department of Fundamental Neurosciences, University of Lausanne, Switzerland

Corresponding author and institutional attribution:
Nicolas Toni, Ph.D.
Department of Fundamental Neurosciences
University of Lausanne,
9, rue du Bugnon, 1005 Lausanne
Switzerland
Tel: ++4121-692-5133
Fax: ++4121-692-5105
Email: Nicolas.toni@unil.ch

Word count:
Abstract: 219
Introduction: 398
Discussion: 1322

The work was received from the Department of Fundamental Neurosciences, University of Lausanne, Switzerland.

Funding: 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. & K.D.)

Acknowledgment: The authors wish to thank the support of Rudolf Kraftsik (B.S., Senior scientist, Department of Fundamental Neurosciences, University of Lausanne, Lausanne, Switzerland) for statistical and image analysis. Images acquisition was performed at the Cellular Imaging Facility of the University of Lausanne (Lausanne, Switzerland).

Running head: Propofol impairs adult neurogenesis

Summary statement: Exposure of adult mice to propofol interferes with the survival and maturation of neurons generated in the adult hippocampus at specific developmental stages.

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. Here we 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 (BrdU) or a retroviral vector expressing the **Green Fluorescent Protein** in dividing cells and their progenies. Eleven or seventeen days following the labeling procedure, animals (n=3 to 5 animals per group) underwent a 6-hour-long propofol anesthesia. Twenty-one days after labeling, we analyzed the survival, differentiation and morphological maturation of adult-born neurons using confocal microscopy.

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 which were 17 day-old at the time of anesthesia, but not of neurons which were 11 day-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.

Introduction

Adult neurogenesis results in the continuous generation of new neurons (referred to as adult-born neurons) which integrate into the brain circuitry throughout adulthood[1]. In most mammalian species, including humans[2], 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[3-5] and inversely, learning tasks and antidepressants increase adult neurogenesis[6,7], which in turn increase learning performances[8]. Adult neurogenesis consists of five discrete processes which are regulated independently: (i) The proliferation of the stem cells, (ii) the differentiation of the progenies into neuronal lineage, (iii) the migration of the new cells into the granule cell layer, (iv) the survival of the new cells, and finally (v) their maturation/integration into the existing neuronal network[9]. Interestingly, neuronal activity plays a strong regulatory role on each of these steps of adult neurogenesis[9-12], thereby enabling a bi-directional regulation of neuronal activity and adult neurogenesis.

During the development of the central nervous system, neuronal maturation is particularly sensitive to external stimuli[13]. 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[14,15]. Adult-born neurons also undergo intense, activity-dependent development and thus may be particularly sensitive to anesthesia exposure. In the present 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 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 hours at two different developmental time-points: 11 or 17 days after BrdU or viral injection (days post-injection, 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[16-18].

Material and methods

Experimental animals

The animals used for this study were eight- to ten-weeks-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 hours, starting by an initial injection of propofol (intraperitoneal, 100 mg/kg body weight, Propofol 1% MCT, Fresenius Kabi, Oberdorf, Switzerland) followed by 5 subsequent injections at 50mg/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, New York, United States of America) blood glucose device, and showed no modification of pH, PCO2, PO2, glucose level and hemoglobin level (Table 1). Control mice were injected with the same regimen and volume of vehicle: 6 injections of soybean oil (Lipofundin MCT/LCT 20%, Braun, Sempach, Switzerland). During anesthesia, animals were kept on a homeothermic blanket system with a temperature probe under the body of one animal in order to maintain body temperature at 37°C (Harvard Apparatus, Holliston, Massachusetts, United States).

BrdU administration and immunohistochemistry

Twenty-four mice were injected intraperitoneally with 50mg/kg of BrdU (Sigma-Aldrich, Buchs, Switzerland) twice, at two hours interval. BrdU intercalates into the nascent DNA strain during cell division and therefore labels dividing cells. Eleven or seventeen days later, mice were either anesthetized for 6 hours (n=5 per time-point), or received three vehicle injections (n=4 mice per group) or 1 vehicle injection (n=3 mice per group). All animals were then perfusion-fixed 21 days after the BrdU injections (Figure 1A). Brains were then dissected, cryoprotected and 40 µm-thick sections were cut on a cryomicrotome and treated for immunohistochemistry as previously described[19]. Briefly, slices were first incubated in 50% formamide/ 50% 2X SSC buffer (2X SSC is 0.3 M NaCl and 0.03 M sodium citrate, pH 7.0) at 65°C for 2 hours, rinsed twice in 2X SSC 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 0.1M phosphate buffer saline containing 0.125% Triton and 3% normal goat serum. The following primary antibodies were used: mouse anti-BrdU (Chemicon International, Dietkon, Switzerland; 1:250) 72 hours 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 (8 to 9 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 of the granule cell layer and multiplied it by the thickness of the sections. For co-localization of BrdU and Neu-N, 20-40

BrdU-immunoreactive cells per animal were examined using confocal microscopy and co-localization 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[17,20]. The concentrated viral solution was prepared using Human Embryonic Kidney 293T cells as a package cell line and collected through ultra-speed 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 µL of virus was injected into the dentate gyrus of either one or two hippocampi of the mouse (stereotaxic coordinates from Bregma: -2mm anteroposterior; -1.75mm lateral; -2.25mm ventral).

Brain sections of one-in-two series were selected for analysis. **Green fluorescent protein** signal was amplified by immunohistochemistry using Chicken anti-green fluorescen protein IgG (Aves Labs, Tigard, **Oregon,** USA; 1:1000) and Dylight 488 goat anti-chicken IgY (Jackson ImmunoResearch Europe ltd., Suffolk, United Kingdom; 1:250). 4,6 Diamidino-2-phenylindole (DAPI) 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 labelling of dendrites

The mice were perfused as described before. Immediately after perfusion, 50 µm-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 previously21.

Confocal microscopy and analysis

Hippocampal sections were imaged using a Leica SP5 AOBS laser scanning microscope. Global views of **Green fluorescent protein**-labeled adult-born neurons were imaged with a 40x oil lens and a z-step of 5 µm, and dendrites were imaged with a 63x oil lens and a z-step of 0.38 µm. Two-dimensional maximum intensity projections of each z-series were created with Fiji software[1]. Dendritic length and spine density **were** measured with Fiji and Trace software[2] (Courtesy John Fiala and Kristen Harris). 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 x π x area/(perimeter)^2.

Statistical analysis

The following morphological 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.

---

[1] Freely available at http://fiji.sc/, last accessed on 11.20.2012

[2] Freely available at http://synapses.clm.utexas.edu/tools/trace/trace.stm, last accessed on 11.20.2012

Hypothesis testing was two-tailed. All analysis was 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, California, USA).** First, Shapiro-Wilk tests were performed on each group of data to test for distribution normality. When the distribution was not normal, non-parametric Kruskal-Wallis test was applied. Otherwise, the analysis was performed using parametric tests (one-way Analysis of Variance 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 is 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 we identified the newly-formed neurons with immunostaining for the neuron-specific marker Neu-N. A total of 24 mice were administered two doses of 50mg/kg of BrdU at two hours interval and were anesthetized for 6 hours at either 11 (n=5) or 17 (n=5) days after BrdU injection (days post-injection, 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: 4 mice received 3 injections of vehicle and 3 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 hours 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, 1 out of 6 hippocampal sections were 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 (Figure 1A). The number of BrdU-immunoreactive cells was significantly reduced in the group anesthetized at 17 dpi as compared to the control group (17 dpi: 18.86 ± 0.57; Ctrl: 23.38 ± 0.77; multiple-comparison Anova test: $F2,21 = 6.14$, $P=0.008$, Bonferroni correction $P<0.01$; Figure 1B) indicating that propofol anesthesia at this developmental time-point decreased the survival of adult-born cells. Co-expression of BrdU and Neu-N was then analyzed on single confocal planes. In all experimental groups, the proportion of BrdU-immunoreactive cells which co-expressed Neu-N was similar between conditions (Ctrl: 94.2 ± 1; 11dpi: 95.4 ± 1.4; 17dpi: 94.7 ± 1.6; one-way Anova: $F2,21=0.21$; $P=0.8$; Figure 1C-D), indicating that propofol anesthesia did not interfere with neuronal

differentiation. The results of the four control groups were comparable and the corresponding data was subsequently pooled. Thus, propofol anesthesia reduced the survival, but not the differentiation of neurons generated 17 days before anesthesia, but not 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[20,22] (Figure 2A). For each neuron (90-200 neurons per mouse, 3-4 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 (Figure 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: $\chi 21 = 39.7$, $p<0.001$; Control vs. 11 dpi: $\chi 21 = 1.21$; $p=0.27$; Figure 2C) and then the frequency distribution of the means per animal of each group (Control vs. 17 dpi: $\chi 21 = 39.7$, degrees of freedom=1, $p<0.001$; Control vs. 11 dpi: $\chi 21 = 1.21$; $p=0.27$; Figure 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[23]. 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, 4-5 mice per group). We observed no morphological difference on mature neurons between control and anesthetized groups, since virtually all neurons displayed dendrites which extended to the tip of the molecular layer (Figure 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, as well as the morphology of their mossy fiber boutons in the CA3 area (consisting of their efferences on pyramidal neurons; Figure 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 ± 0.06 spines/µm in control animals vs 0.42 ± 0.08 spines/µm in animals anesthetized at 17 days; spine density in the middle third of the molecular layer: 0.71 ± 0.06 spines/µm in control animals vs. 0.79 ± 0.11 spines/µm in animals anesthetized at 17 days. 900-3000 spines, 24-78 neurons and 3-4 mice per condition; Kruskal-Wallis test, p=0.44 and p=0.33, respectively; Figure 3A). We examined the spine density on control neurons of the external granule cell layer which 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, 3-5 mice per condition, spine density in the inner third of the molecular layer: 1.66 ± 0.22 spines/µm in control animals vs. 1.58 ± 0.13 spines/µm in animals anesthetized at 17 days; spine density in the middle third of the molecular layer: 1.51 ± 0.19 spines/µm in control animals vs.1.40 ± 0.12 spines/µm in animals anesthetized at 17 days; spine density of control dendrites in the middle third of the molecular layer: 1.76 ± 0.03 spines/µm in control animals vs. 1.54 ± 0.13 spines/µm in animals anesthetized at 17 days; Unpaired t-test, p=0.74, p=0.64 and p=0.28 respectively; Figure 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 (3-4 mice per condition; 11-21 boutons per mouse; Area: 5.12 ± 0.6 µm2 in control animals vs. 5.25 ± 0.6 µm2 in animals anesthetized at 11 dpi and 4.8 ± 0.2 µm2 in animals anesthetized at 17 dpi, Kruskal-Wallis test: p=0.70; Circularity: 0.49 ± 0.03 in control animals vs. 0.49 ± 0.04 µm2 in animals anesthetized at 11 dpi and 0.56 ± 0.03 in animals anesthetized at 17 dpi, one-way Analysis of Variance: p=0.27; Number of extensions per

bouton: 2.7 ± 0.47 in control animals vs. 2.49 ± 0.3 µm2 in animals anesthetized at 11 dpi and 2.6 ± 0.29 in animals anesthetized at 17 dpi, Kruskal-Wallis test: p=0.97; Figure 3D-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 the present 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, since propofol exposure led to a significant decrease in the survival of neurons which were 17-day-old but not 11-day-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 while 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. Since 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 suggest 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 indicate that adult-born neurons play an important role in information processing underlying spatial cognition and learning[24-26]. 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[11,12,27-30]. In this context, since the majority of general anesthetics are powerful potentiators of neurotransmission via **gamma-aminobutyric acid A (GABAA)** receptors[31], exposure to these drugs could impede the physiological neural activity patterns required for the maturation and integration of adult-born neurons. To elucidate this hypothesis, we focused on propofol since 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[32]. While 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., investigated how anesthesia interferes with neuronal proliferation, differentiation and survival[32]. 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- as compared to 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[12]. 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[33]. 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[12]. This supports the view that GABA-receptor activity is required for the proper maturation of adult-born neurons[11,27-30,34] and that the exposure of

immature neurons to propofol may induce maturation defects. However, the maturation of adult-born neurons involves several other developmental steps, such as a changes in **N-methyl-D-aspartic acid (NMDA)** receptor composition35 and of synaptic connectivity36. 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-17dpi 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 development37 and the observation that GABA-receptor activity in adult-born neurons is necessary for the development of dendritic tree12. An alternative explanation is that propofol anesthesia induces the selective loss of neurons with larger dendritic extensions in the P-17dpi 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[21,37,38]. 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 present study, reduces the overall axo-spinous innervation on new neurons. In a previous experiment, we showed that inhibiting neuronal activity specifically on adult-born neurons results in an 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 neurons10. Thus, both network and cell-autonomous activity may play a critical role for the survival and maturation of adult-born neurons and a pharmacological 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 which 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 function3,24,39,40 and in learning8,41. Interestingly, recent results indicate that ablating neurogenesis in a specific time-window results in long-lasting learning impairment3,39-42. Furthermore, learning induces structural plasticity of adult-born neurons, which in turn contribute to the network remodeling induced by learning[26]. 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[43-45]. In these experiments, isoflurane/nitrous oxide anesthesia impaired acquisition of new tasks either in the young or in the aged rats, while retrieval of consolidated memory was only reduced in aged animals. More recent data, however, revealed no effect of a 2-hour-long propofol exposure on spatial working memory as tested by the 12-arm radial maze test[46]. 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 cells12. Given the important role of adult hippocampal neurogenesis in

the life-long acquisition of new memories, our findings might also provide potential mechanisms underlying impaired cognitive function in the postoperative period.

References

1. Altman J, Das GD: Post-natal origin of microneurones in the rat brain. Nature 1965; 207: 953-6

2. Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH: Neurogenesis in the adult human hippocampus. Nat Med 1998; 4: 1313-7

3. Saxe MD, Battaglia F, Wang JW, Malleret G, David DJ, Monckton JE, Garcia AD, Sofroniew MV, Kandel ER, Santarelli L, Hen R, Drew MR: Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. Proc Natl Acad Sci U S A 2006; 103: 17501-6

4. Shors TJ, Miesegaes G, Beylin A, Zhao M, Rydel T, Gould E: Neurogenesis in the adult is involved in the formation of trace memories. Nature 2001; 410: 372-6

5. Massa F, Koehl M, Wiesner T, Grosjean N, Revest JM, Piazza PV, Abrous DN, Oliet SH: Conditional reduction of adult neurogenesis impairs bidirectional hippocampal synaptic plasticity. Proc Natl Acad Sci U S A 2011; 108: 6644-9

6. Gould E, Beylin A, Tanapat P, Reeves A, Shors TJ: Learning enhances adult neurogenesis in the hippocampal formation. Nat Neurosci 1999; 2: 260-5

7. Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, Weisstaub N, Lee J, Duman R, Arancio O, Belzung C, Hen R: Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science 2003; 301: 805-9

8. van Praag H, Christie BR, Sejnowski TJ, Gage FH: Running enhances neurogenesis, learning, and long-term potentiation in mice. Proc.Natl.Acad.Sci.U S.A. 1999; 96: 13427-31

9. Toni N, Sultan S: Synapse formation on adult-born hippocampal neurons. Eur J Neurosci 2011; 33: 1062-8

10. Tashiro A, Sandler VM, Toni N, Zhao C, Gage FH: NMDA-receptor-mediated, cell-specific integration of new neurons in adult dentate gyrus. Nature 2006; 442: 929-33

11. Ge S, Pradhan DA, Ming GL, Song H: GABA sets the tempo for activity-dependent adult neurogenesis. Trends Neurosci 2007; 30: 1-8

12. Ge S, Goh EL, Sailor KA, Kitabatake Y, Ming GL, Song H: GABA regulates synaptic integration of newly generated neurons in the adult brain. Nature 2006; 439: 589-93

13. Hensch TK: Critical period regulation. Annu Rev Neurosci 2004; 27: 549-79

14. Loepke AW, Soriano SG: An assessment of the effects of general anesthetics on developing brain structure and neurocognitive function. Anesth Analg 2008; 106: 1681-707

15. Vutskits L: Anesthetic-related neurotoxicity and the developing brain: shall we change practice? Paediatr Drugs 2012; 14: 13-21

16. Toni N, Laplagne DA, Zhao C, Lombardi G, Ribak CE, Gage FH, Schinder AF: Neurons born in the adult dentate gyrus form functional synapses with target cells. Nat Neurosci 2008; 11: 901-7

17. Toni N, Teng EM, Bushong EA, Aimone JB, Zhao C, Consiglio A, van Praag H, Martone ME, Ellisman MH, Gage FH: Synapse formation on neurons born in the adult hippocampus. Nat Neurosci 2007; 10: 727-34

18. Laplagne DA, Esposito MS, Piatti VC, Morgenstern NA, Zhao C, van Praag H, Gage FH, Schinder AF: Functional convergence of neurons generated in the developing and adult hippocampus. PLoS Biol 2006; 4: 2349-60

19. Thuret S, Toni N, Aigner S, Yeo GW, Gage FH: Hippocampus-dependent learning is associated with adult neurogenesis in MRL/MpJ mice. Hippocampus 2009; 19: 658-69

20. van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH: Functional neurogenesis in the adult hippocampus. Nature 2002; 415: 1030-4

21. Briner A, De Roo M, Dayer A, Muller D, Habre W, Vutskits L: Volatile anesthetics rapidly increase dendritic spine density in the rat medial prefrontal cortex during synaptogenesis. Anesthesiology 2010; 112: 546-56

22. Zhao C, Teng EM, Summers RG, Jr., Ming GL, Gage FH: Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus. J Neurosci 2006; 26: 3-11

23. Kempermann G, Gast D, Kronenberg G, Yamaguchi M, Gage FH: Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice. Development 2003; 130: 391-9

24. Schmidt-Hieber C, Jonas P, Bischofberger J: Enhanced synaptic plasticity in newly generated granule cells of the adult hippocampus. Nature 2004; 429: 184-7

25. Marin-Burgin A, Mongiat LA, Pardi MB, Schinder AF: Unique processing during a period of high excitation/inhibition balance in adult-born neurons. Science 2012; 335: 1238-42

26. Tronel S, Fabre A, Charrier V, Oliet SH, Gage FH, Abrous DN: Spatial learning sculpts the dendritic arbor of adult-born hippocampal neurons. Proc Natl Acad Sci U S A 2010; 107: 7963-8

27. Tozuka Y, Fukuda S, Namba T, Seki T, Hisatsune T: GABAergic excitation promotes neuronal differentiation in adult hippocampal progenitor cells. Neuron 2005; 47: 803-15

28. Sun B, Halabisky B, Zhou Y, Palop JJ, Yu G, Mucke L, Gan L: Imbalance between GABAergic and Glutamatergic Transmission Impairs Adult Neurogenesis in an Animal Model of Alzheimer's Disease. Cell Stem Cell 2009; 5: 624-33

29. Li G, Bien-Ly N, Andrews-Zwilling Y, Xu Q, Bernardo A, Ring K, Halabisky B, Deng C, Mahley RW, Huang Y: GABAergic interneuron dysfunction impairs hippocampal neurogenesis in adult apolipoprotein E4 knockin mice. Cell Stem Cell 2009; 5: 634-45

30. Schinder AF, Morgenstern NA: Preview. Adult neurogenesis is altered by GABAergic imbalance in models of Alzheimer's disease. Cell Stem Cell 2009; 5: 573-4

31. Rudolph U, Antkowiak B: Molecular and neuronal substrates for general anaesthetics. Nat Rev Neurosci 2004; 5: 709-20

32. Stratmann G, Sall JW, Bell JS, Alvi RS, May LV, Ku B, Dowlatshahi M, Dai R, Bickler PE, Russell I, Lee MT, Hrubos MW, Chiu C: Isoflurane does not affect brain cell death, hippocampal neurogenesis, or long-term neurocognitive outcome in aged rats. Anesthesiology 2010; 112: 305-15

33. Ganguly K, Schinder AF, Wong ST, Poo M: GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. Cell 2001; 105: 521-32

34. Song J, Zhong C, Bonaguidi MA, Sun GJ, Hsu D, Gu Y, Meletis K, Huang ZJ, Ge S, Enikolopov G, Deisseroth K, Luscher B, Christian KM, Ming GL, Song H: Neuronal circuitry mechanism regulating adult quiescent neural stem-cell fate decision. Nature 2012; 489: 150-4

35. Ge S, Yang CH, Hsu KS, Ming GL, Song H: A critical period for enhanced synaptic plasticity in newly generated neurons of the adult brain. Neuron 2007; 54: 559-66

36. Vivar C, Potter MC, Choi J, Lee JY, Stringer TP, Callaway EM, Gage FH, Suh H, van Praag H: Monosynaptic inputs to new neurons in the dentate gyrus. Nat Commun 2012; 3: 1107

37. Briner A, Nikonenko I, De Roo M, Dayer A, Muller D, Vutskits L: Developmental Stage-dependent persistent impact of propofol anesthesia on dendritic spines in the rat medial prefrontal cortex. Anesthesiology 2011; 115: 282-93

38. De Roo M, Klauser P, Briner A, Nikonenko I, Mendez P, Dayer A, Kiss JZ, Muller D, Vutskits L: Anesthetics rapidly promote synaptogenesis during a critical period of brain development. PLoS One 2009; 4: 1-9

39. Clelland CD, Choi M, Romberg C, Clemenson GD, Jr., Fragniere A, Tyers P, Jessberger S, Saksida LM, Barker RA, Gage FH, Bussey TJ: A functional role for adult hippocampal neurogenesis in spatial pattern separation. Science 2009; 325: 210-3

40. Imayoshi I, Sakamoto M, Ohtsuka T, Takao K, Miyakawa T, Yamaguchi M, Mori K, Ikeda T, Itohara S, Kageyama R: Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain. Nat Neurosci 2008; 11: 1153-61

41. Deng W, Aimone JB, Gage FH: New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? Nat Rev Neurosci 2010; 11: 339-50

42. Lemaire V, Tronel S, Montaron MF, Fabre A, Dugast E, Abrous DN: Long-lasting plasticity of hippocampal adult-born neurons. J Neurosci 2012; 32: 3101-8

43. Culley DJ, Baxter M, Yukhananov R, Crosby G: The memory effects of general anesthesia persist for weeks in young and aged rats. Anesth Analg 2003; 96: 1004-9

44. Culley DJ, Baxter MG, Crosby CA, Yukhananov R, Crosby G: Impaired acquisition of spatial memory 2 weeks after isoflurane and isoflurane-nitrous oxide anesthesia in aged rats. Anesth Analg 2004; 99: 1393-7

45. Culley DJ, Baxter MG, Yukhananov R, Crosby G: Long-term impairment of acquisition of a spatial memory task following isoflurane-nitrous oxide anesthesia in rats. Anesthesiology 2004; 100: 309-14

46. Lee IH, Culley DJ, Baxter MG, Xie Z, Tanzi RE, Crosby G: Spatial memory is intact in aged rats after propofol anesthesia. Anesth Analg 2008; 107: 1211-5

Figure Legends

Figure 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 either 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 ± 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 which expressed the neuronal marker Neu-N. Anova between groups $F(2,21)=0.21$, P=0.81.

Figure 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 17 dpi (A3). **B**. Schematic illustration of the morphometric analyses: dendritic extension = a / b x 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). ML: molecular layer; GCL: granule cell layer. **C**. Histogram showing the relative frequency of neurons per dendritic extension range. Chi-Square test; Control vs 17 dpi: p<0.001 (***); Control vs 11 dpi: P=0.27, n=3 to 4.

Figure 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 to 4. **B**. Scatter plot of the dendritic spine density in the inner and middle thirds of the molecular layer in control neurons, i.e. granule neurons from the outer third of the granule cell layer, or random dendrites labeled with DiI. Unpaired two-tailed Student's t-test: P>0.1, n=2 to 5. Data is expressed as mean values ± 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.

Figure 1

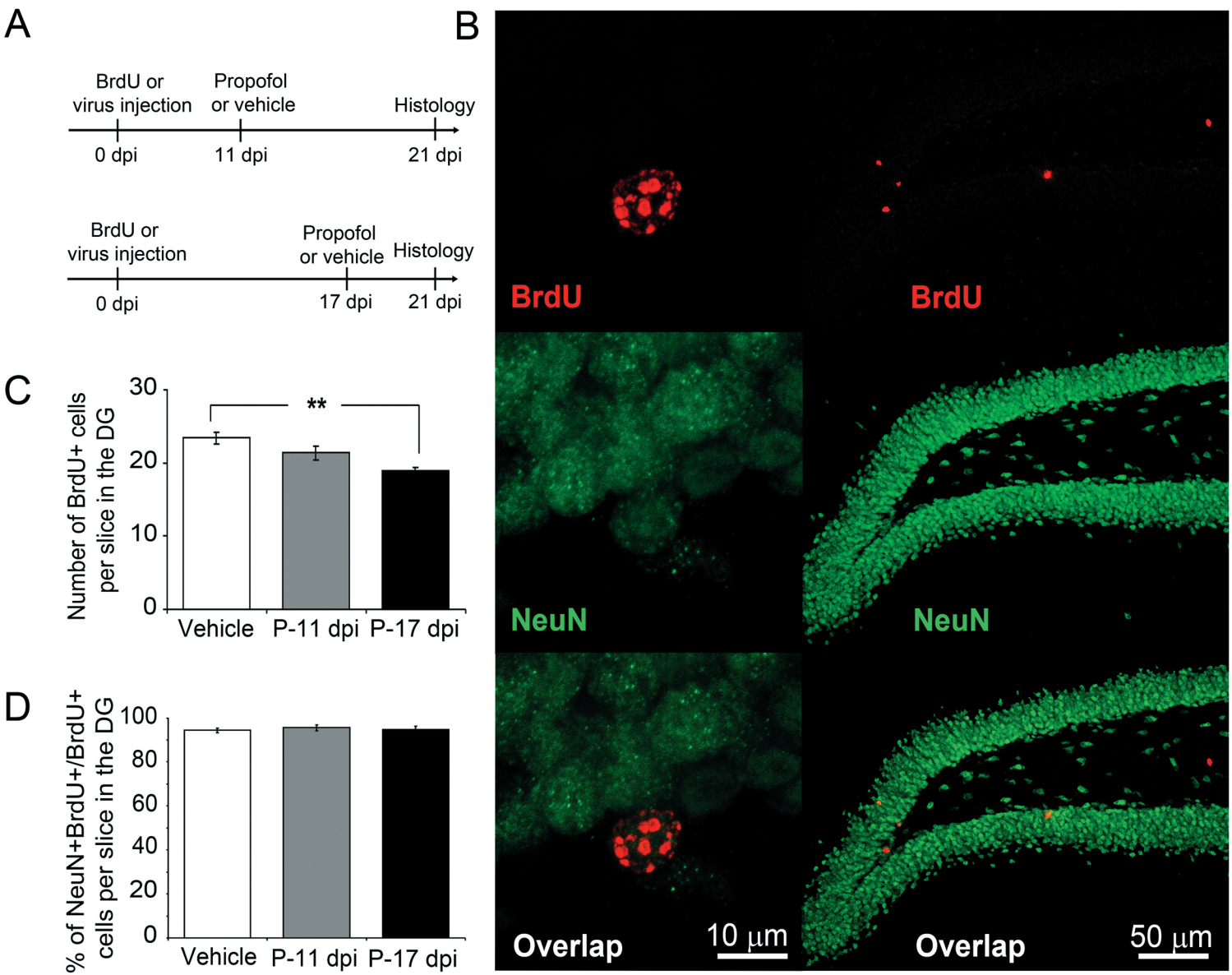

A
BrdU or virus injection
Propofol or vehicle
Histology
0 dpi
11 dpi
21 dpi
BrdU or virus injection
Propofol or vehicle
Histology
0 dpi
17 dpi
21 dpi
B
BrdU
BrdU
NeuN
NeuN
Overlap
10 μm
Overlap
50 μm
C
Number of BrdU+ cells per slice in the DG
30
20
10
0
**
Vehicle
P-11 dpi
P-17 dpi
D
% of NeuN+BrdU+/BrdU+ cells per slice in the DG
100
80
60
40
20
0
Vehicle
P-11 dpi
P-17 dpi

Figure 2

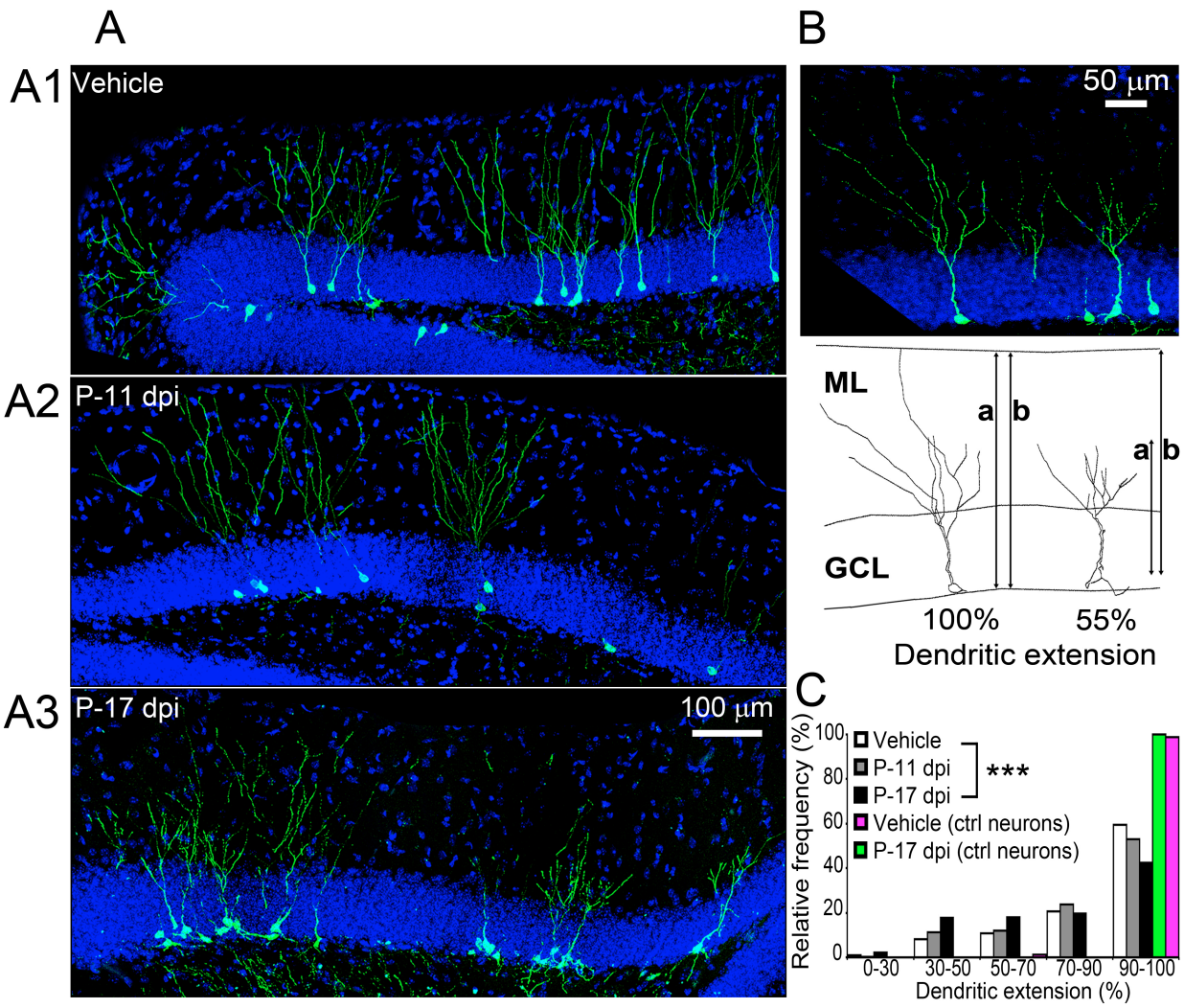
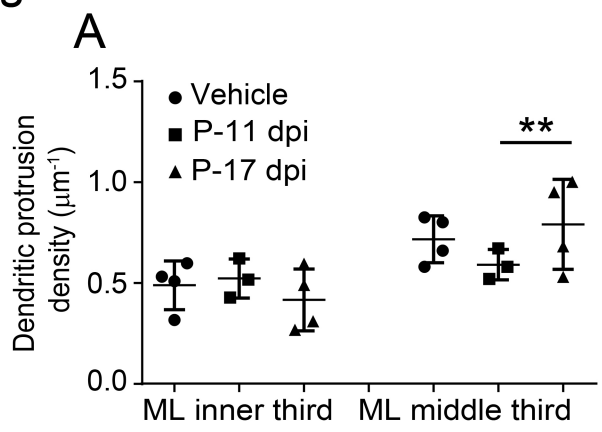
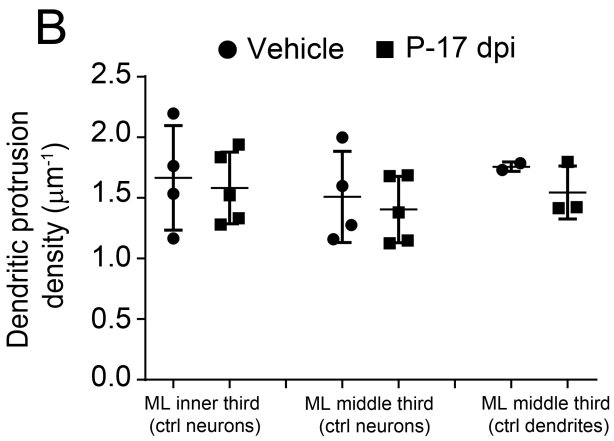
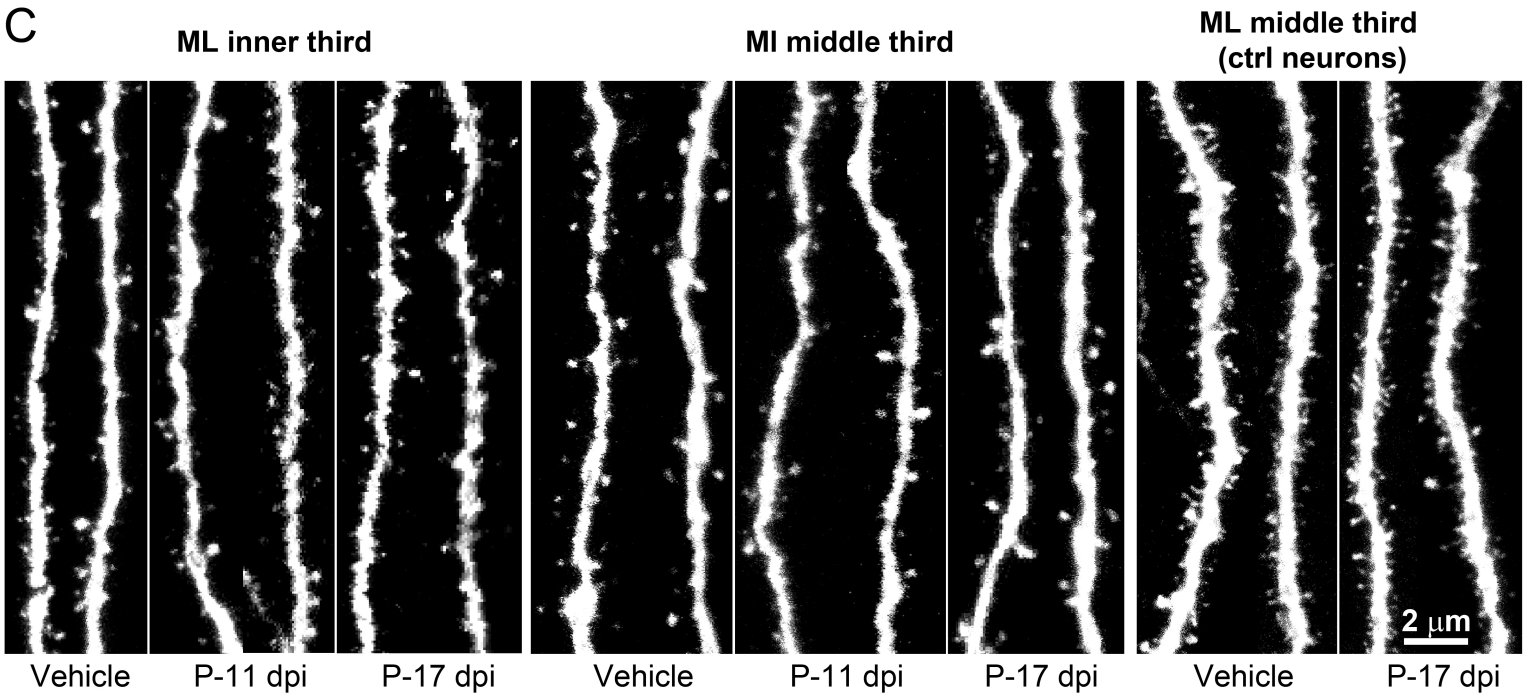
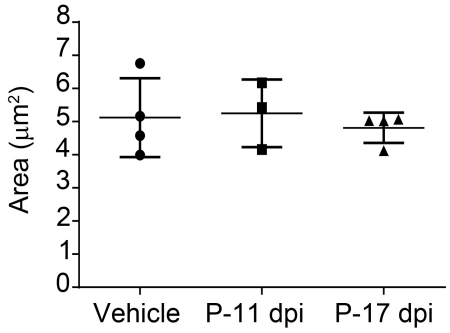

A
A1
Vehicle
A2
P-11 dpi
A3
P-17 dpi
100 μm
B
50 μm
ML
GCL
a
b
a
b
100%
55%
Dendritic extension
C
Relative frequency (%)
100
80
60
40
20
0
Vehicle
P-11 dpi
P-17 dpi
***
Vehicle (ctrl neurons)
P-17 dpi (ctrl neurons)
0-30
30-50
50-70
70-90
90-100
Dendritic extension (%)

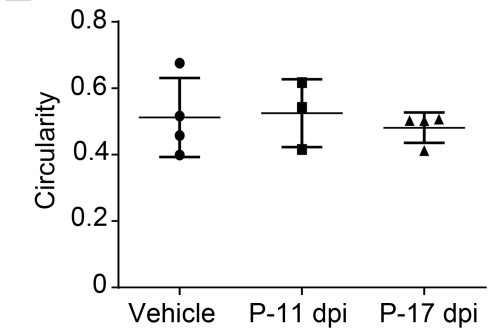
Figure 3

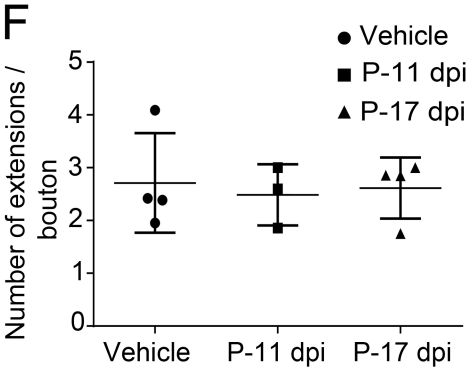

A
Vehicle
P-11 dpi
P-17 dpi
Dendritic protrusion density (μm-1)
1.5
1.0
0.5
0.0
**
ML inner third
ML middle third

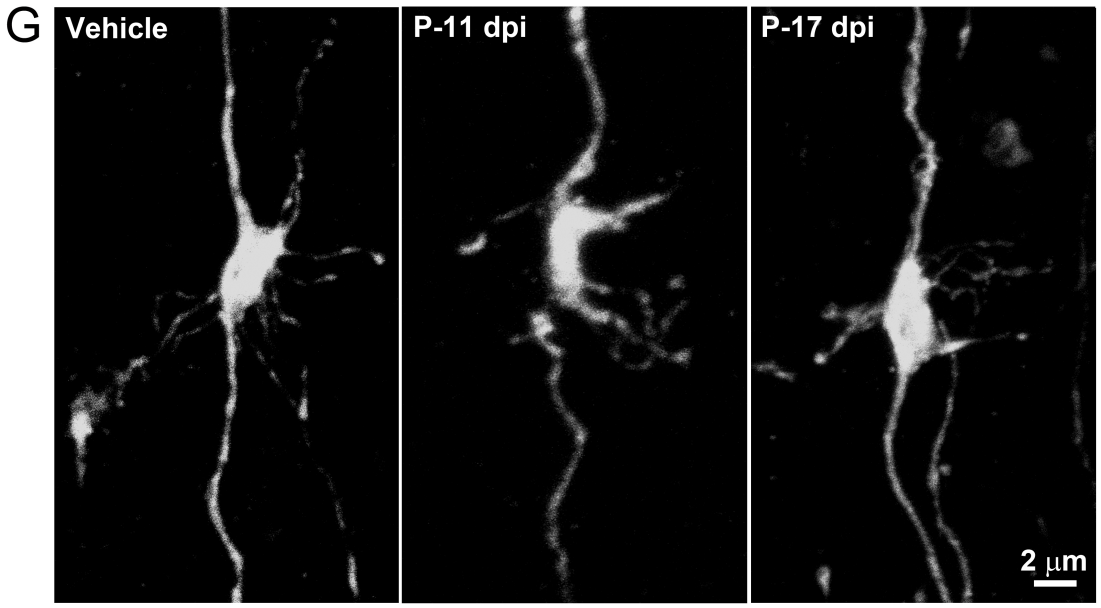

B
Vehicle
P-17 dpi
Dendritic protrusion density (μm-1)
2.5
2.0
1.5
1.0
0.5
0.0
ML inner third (ctrl neurons)
ML middle third (ctrl neurons)
ML middle third (ctrl dendrites)


C
ML inner third
Ml middle third
ML middle third (ctrl neurons)
2 μm
Vehicle
P-11 dpi
P-17 dpi
Vehicle
P-11 dpi
P-17 dpi
Vehicle
P-17 dpi


D
Area (μm2)
8
7
6
5
4
3
2
1
0
Vehicle
P-11 dpi
P-17 dpi


E
Circularity
0.8
0.6
0.4
0.2
0
Vehicle
P-11 dpi
P-17 dpi


F
Vehicle
P-11 dpi
P-17 dpi
Number of extensions / bouton
5
4
3
2
1
0
Vehicle
P-11 dpi
P-17 dpi


G
Vehicle
P-11 dpi
P-17 dpi
2 μm

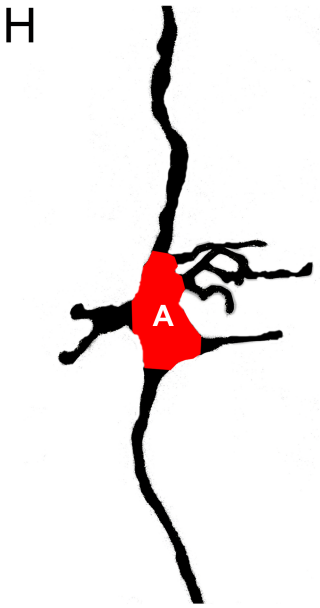

H
A

Table 1

Blood physiological parameters

	Vehicle	Propofol
pH	7.35 ± 0.04	7.35 ± 0.04
pCO_2 (kPa)	6.76 ± 0.4	6.55 ± 0.6
Glucose ($mg.dL^{-1}$)	9.75 ± 0.6	9.48 ± 1
Hemoglobin ($g.dL^{-1}$)	10.1 ± 0.6	9.8 ± 0.6

Table 1. Blood physiological parameters remain within normal range during anesthesia. After 6 hours of propofol anesthesia or vehicle injection, blood parameters were similar between groups ($p>0.1$, unpaired two-tailed Student's t-test, $n=4$).
Data is expressed as mean values + SEM