

Developmental Stage-dependent Persistent Impact of Propofol Anesthesia on Dendritic Spines in the Rat Medial Prefrontal Cortex

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ABSTRACT

Background: Recent observations demonstrate that anesthetics rapidly impair synaptogenesis during neuronal circuitry development. Whether these effects are lasting and depend on the developmental stage at which these drugs are administered remains, however, to be explored.

Methods: Wistar rats received propofol anesthesia at defined developmental stages during early postnatal life. The acute and long-term effects of these treatments on neuronal cytoarchitecture were evaluated by Neurolucida and confocal microscopy analysis after iontophoretic injections of Lucifer Yellow into layer 5 pyramidal neurons in the medial prefrontal cortex. Quantitative electron microscopy was applied to investigate synapse density.

Results: Layer 5 pyramidal neurons of the medial prefrontal cortex displayed intense dendritic growth and spinogenesis during the first postnatal month. Exposure of rat pups to propofol at postnatal days 5 and 10 significantly decreased dendritic spine density, whereas this drug induced a significant increase in spine density when administered at

What We Already Know about This Topic

- General anesthetics affect early synaptic plasticity in animal models, such as increasing or decreasing dendritic spine density

What This Article Tells Us That Is New

- Propofol anesthesia in rats at defined developmental stages resulted in reduced dendritic spine density in the early postnatal period and increased spine density and synapse formation with later exposure
- These propofol-induced changes could lead to persistent changes in neuronal circuitry and function

postnatal days 15, 20, or 30. Quantitative electron microscopy revealed that the propofol-induced increase in spine density was accompanied by a significant increase in the number of synapses. Importantly, the propofol-induced modifications in dendritic spine densities persisted up to postnatal day 90.

Conclusion: These new results demonstrate that propofol anesthesia can rapidly induce significant changes in dendritic spine density and that these effects are developmental stage-dependent, persist into adulthood, and are accompanied by alterations in synapse number. These data suggest that anesthesia in the early postnatal period might permanently impair circuit assembly in the developing brain.

THE capacity of a neuron to innervate and function within a network is mediated *via* specialized cell junctions known as synapses. During central nervous system development, each neuron establishes thousands of synaptic contacts with other neurons, and the appropriate function of neuronal networks is primarily based on this high level of interconnectivity between these cells. Synaptogenesis is thus a key step during brain circuitry assembly, and inappropriate synapse formation or structure is thought to underlie a variety of neurodevelopmental disorders.^{1,2} Understanding how synapse formation is regulated under normal and pathologic conditions is, therefore, of utmost interest. Neuronal activity appears to be a key factor during synaptogenesis.³ Accumulating experimental works demonstrate that, during critical periods of development, both environmental, genetic, and pharmacologic interference with physiologic neuronal activity can markedly

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Address correspondence to Dr. Vutskits: Department of Anesthesiology, Pharmacology and Intensive Care, University Hospital of Geneva, 4, rue Gabrielle-Perret-Gentil.1211 Geneva 4, Switzerland. laszlo.vutskits@unige.ch. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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and permanently alter wiring patterns and, thereby, information processing in the central nervous system.^{4–6}

Synaptogenesis in the human neocortex occurs between the third trimester of gestation and the first few years of postnatal life.^{7–9} Importantly, the most intense phase of synaptic growth takes place during the first 6 postnatal months when the number of synaptic contacts doubles in most cortical regions.¹⁰ In the rodent cerebral cortex, cortical synaptogenesis is restricted to the early postnatal period with a peak synaptic growth occurring between the second and fourth postnatal weeks. During these 3 weeks, there is a more than 10-fold increase in the number of excitatory synaptic contacts and an approximately threefold increase in the number of γ -aminobutyric acid—mediated (GABAergic) inhibitory synapses in the cerebral cortex.^{11–13}

Increasing experimental evidence indicates an important role of GABAergic and glutamatergic signaling during synaptogenesis.^{5,6,14} Because most currently used general anesthetics potentiate neurotransmission *via* the γ -aminobutyric acid receptor type (GABA_A) receptor complex or by inhibition of neurotransmitter release,¹⁵ an intriguing possibility is that exposure to these drugs during critical periods of synaptic development might interfere with neural circuitry assembly. In line with this hypothesis, recent studies, performed at early stages of cortical synaptogenesis in 5- to 7-day-old rat pups, have demonstrated decreased synaptic density in the hippocampal formation after administration of general anesthetics in these animals.^{16,17} In contrast to this anesthetic-induced decrease in synaptic density at earlier stages of development, we have recently demonstrated that exposure of rodent pups to either intravenous or volatile anesthetics at postnatal day (PND) 15 rapidly promoted dendritic spine and synaptogenesis in several brain areas.^{18,19} These seemingly contradictory observations suggest that the effects of anesthetics on synaptogenesis are multifaceted and might depend on the developmental stage at which organisms are exposed to these drugs. To clarify this issue, a systematic evaluation of how anesthetics impact on synaptogenesis at distinct time points of central nervous system development is necessary. In these experiments, long-term follow-up of synapse density would also help to determine whether anesthetics-induced alterations of synaptogenesis during the early postnatal period would lead to permanent rewiring of neural circuitry. Last but not least, applying different lengths of anesthesia maintenance will provide important information about the time of exposure necessary to induce and retain changes in synaptic structures by these drugs.

The current study was designed to investigate these aforementioned important issues. By focusing on layer 5 pyramidal neurons of the rat medial prefrontal cortex (mPFC), we first characterized dendritic arbor and spine development of these cells between PND 5 and 30, a period corresponding to synaptogenesis in these animals. We then systematically evaluated the acute and long-term effects of a single dose as well as a 6-h-long propofol anesthesia protocol on dendritic

spines, representing excitatory synaptic contacts, at distinct stages of synaptogenesis during development. These experiments revealed that although up to a 6-h-long propofol exposure does not affect dendritic arbor development, even a single dose of this drug can rapidly induce significant and permanent changes in dendritic spine and synapse density in the mPFC, and that this effect is dependent on the developmental stage at which this drug was applied. These results strongly suggest that exposure to propofol can induce substantial changes in synapse number, thereby shaping cortical connectivity in animals exposed to this drug during critical periods of synaptogenesis.

Materials and Methods

The experimental protocol was reviewed and approved by the Ethics Committee of the University Medical Center of Geneva and by the Cantonal Veterinary Office, Geneva, Switzerland. Animals were group-housed and bred in the animal facilities of the University of Geneva Medical School under light- (12 h light/dark cycle) and temperature-controlled ($22 \pm 2^\circ\text{C}$) conditions. Food and water were available *ad libitum*. Every effort was made to minimize the number of animals used and their suffering. Five- to 90-day-old Wistar rats were used for all experiments. Both male and female animals were considered up to 20 days of age, whereas only male rats were analyzed after this developmental stage.

Anesthesia Procedure

General anesthesia was induced by intraperitoneal injection of propofol (40 mg/kg). At this concentration, this drug induced deep sedation (absence of the righting reflex more than 10 s) for approximately 1 h. Subsequent doses of propofol (20 mg/kg) allowed anesthesia to be maintained for approximately 1 additional hour. Thus, to provide a 6-h-long anesthesia, animals received a total of five intraperitoneal injections of anesthetics at 60-min intervals. Control sham-treated animals received five intraperitoneal injections of intralipid at 60-min intervals. They also underwent the same maternal separation and handling as anesthetized animals and were kept in individual cages for the duration of the experimental procedure. Body temperature was monitored and maintained between 37 and 38°C by means of a heating pad (Harvard Apparatus, Holliston, MA). In a distinct set of experiments, blood gas values, through puncture of the right atrium, were determined in PND 5 animals after a 6-h-long propofol anesthesia to ensure adequacy of spontaneous respiration and other physiologic parameters. In agreement with our previous observations,¹⁹ these experiments confirmed that this treatment paradigm induced no important respiratory or metabolic derangements in these animals (data not shown). Blood gas analysis was performed with the handheld i-STAT analyzer (Abbott AG, Baar, Switzerland), blood glucose with Bayer Ascensia Contour (Bayer Healthcare, Tarrytown, NY).

Iontophoretic Post Hoc Single Cell Injections and Tissue Processing

According to the experimental protocol (see Results), animals were sacrificed by an overdose of pentobarbital (120 mg/kg intraperitoneally) and perfused transcardially with a 4% paraformaldehyde, 0.125% glutaraldehyde (pH 7.4) solution. Iontophoretic injections, tissue processing, and immunohistochemistry were performed as described before.¹⁹ The medial prefrontal cortex was identified as the area between the forceps minor corpus callosum and the midline, approximately 3.4 mm rostral from bregma. Layer 5 pyramidal neurons were identified by their large size and apical dendrite.

Analysis of Neuronal Cytoarchitecture and Dendritic Tree and Spines

Only pyramidal neurons lying within layer 5 of the mPFC with proper filling of distal dendritic tips were included into the analysis. Reconstruction of three-dimensional dendritic structure was done on a computer-based NeuroLucida system (MicroBrightfield, Williston, VT) with a 40 \times objective on a Nikon microscope (Nikon Corporation, Tokyo, Japan). In each experimental group, total dendritic length, number of branching points, and Sholl distribution²⁰ for both basal and apical dendritic arbors were quantified by an observer blind to the experimental conditions. An LSM 510 meta confocal microscope (Carl-Zeiss, Göttingen, Germany) equipped with a 63 \times oil-immersion objective was used for dendritic spine analysis. Spine analysis was performed on acquired stacks of images using a homemade plug-in written for OsiriX software (Pixmeo, Geneva, Switzerland). This plug-in allows precise spine quantification, individual tagging, and measurement in three dimensions by scrolling through the z axis. We defined spines as all structures emerging from the dendrites that were also longer than 0.4 μ m and for which we could distinguish an enlargement at the tip (spine head). The largest spine head widths were measured in the xy axis on the z-image corresponding to the central axis of the spine head.

Note that for illustration purposes, images presented in figures are maximum intensity projections of z stacks with volume rendering, further treated with a gaussian blur filter.

Electron Microscopy

Intracardial perfusion in rat pups was done with 4% paraformaldehyde and 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). After an overnight postfixation in the same fixative, the brains were cut on a vibratome (150- μ m thick) and embedded in EPON resin (Fluka, Chemicals, Gillingham, United Kingdom). Semithin sections (2 μ m) were stained with methylene blue and the pyramids for ultrathin cutting were trimmed in the layer V of prefrontal cortex. Ultrathin sections (60 nm, ultratome Ultracut-E; Reichert-Jung, Leica, Heerbrugg, Switzerland) were collected on single-slot Formvar-coated grids and stained with 5% uranyl acetate and lead citrate. The electron microscopic images of the cortical neuropil were taken randomly, at a magnification of \times 9700, on several consecutive

sections in the places devoid of big processes and cell bodies, using the transmission electron microscope Tecnai G2 12 (FEI Company, Hillsboro, OR) equipped with a digital camera (Mega View III, Soft Imaging Systems, Olympus, Tokyo, Japan). Synapses were defined by the presence of postsynaptic density facing a presynaptic bouton with at least three synaptic vesicles. Excitatory synapses were distinguished by morphologic criteria: pronounced postsynaptic density facing a presynaptic bouton containing round synaptic vesicles with presynaptic and postsynaptic parts separated by a widened synaptic cleft. The number of spine and shaft synapses was estimated using the physical disector method on a series of consecutive serial sections.²¹ Spine synapses were distinguished by the presence of a spine head with a postsynaptic density facing a presynaptic bouton. In shaft synapses, postsynaptic densities were localized directly on dendritic shafts. In total, 192 disectors spanning the volume of 581.76 μ m³ and 194 disectors with the total volume of 587.82 μ m³ were analyzed in control and anesthetized groups, respectively.

Statistics

All statistics are given with the mean and the SD. Normality was tested for each distribution (D'Agostino and Pearson test), and α was set to 5% for all tests. At each developmental stage tested, control conditions were compared with the mean spine density obtained from single bolus as well as 6-h-long propofol-exposed individual cells, distinguishing either between basal and apical dendrites or between spine head diameter-defined groups. For this, we performed for each group one-way ANOVA with Bonferroni correction *post hoc* tests using Prism Software, Version 5.0a (GraphPad Software Inc., La Jolla, CA). Where appropriate, a two-tailed Student *t* test was performed and $P < 0.05$ was considered statistically significant.

Results

Intense Dendritic Growth and Spinogenesis of Layer 5 mPFC Pyramidal Neurons in the Early Postnatal Period

Because the peak period of synaptogenesis in the rodent cerebral cortex is reported to occur between the second and fourth weeks of postnatal life,¹³ we first explored physiologic patterns of dendritic arbor and spine development of layer 5 pyramidal neurons in the mPFC at distinct developmental time points extending from PND 5 to PND 30. Intracellular iontophoretic injection of Lucifer Yellow into these cells, allowing complete filling of apical and basal dendrites with dendritic spines,¹⁹ revealed an approximately 3.5-fold increase in total dendritic length of either apical (+363 \pm 24%) or basal (+365 \pm 22%) dendrites during this period (fig. 1A–B). Analysis of the number of branching points showed a highly significant increase between PND 5 and PND 10 in either apical (13 \pm 3 *vs.* 29 \pm 4; $P = 0.0006$) or basal (13 \pm 1 *vs.* 21 \pm 1; $P < 0.0001$) dendritic arbor complexity, with no further increase after PND 10 (fig. 1C).

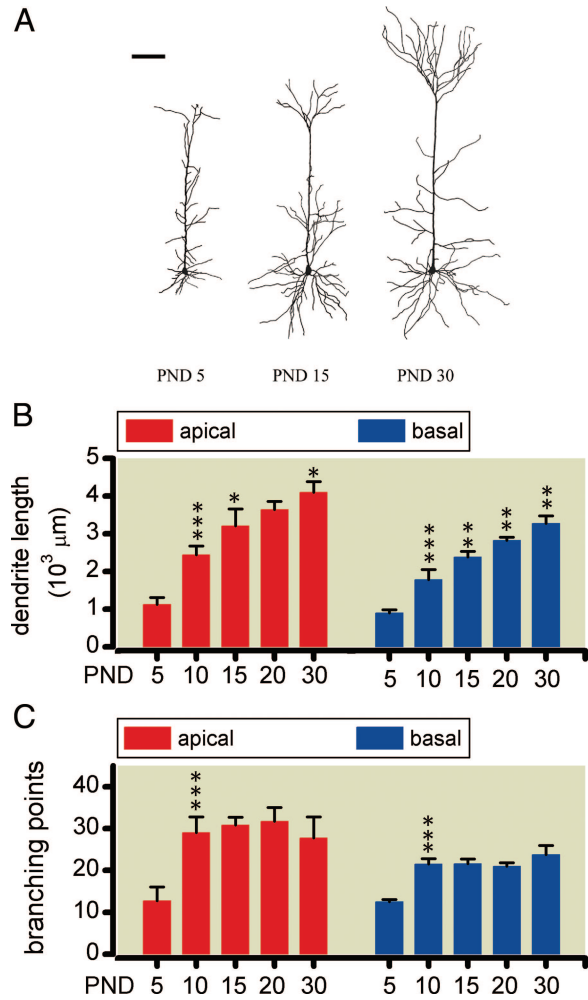


Fig. 1. Dendritic arbor development of layer 5 pyramidal neurons in the medial prefrontal cortex of Wistar rats during the first month of postnatal life. (A) Representative Neurolucida reconstructions of Lucifer Yellow-injected pyramidal neurons in layer 5 of the medial prefrontal cortex at postnatal days 5, 10, and 30 showing the temporal evolution of dendritic arborization pattern in these cells during the first postnatal month. (B) Quantitative analysis describing the temporal evolution of apical (red columns) and basal (blue columns) dendritic lengths and (C) branching points during this same period. Results are expressed as mean ± SD. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$, one-way ANOVA with Bonferroni correction posttests performed between subsequent age groups in either basal or apical tree, $n = 4$ animals for age groups PND 5, 10, 20 and $n = 5$ animals for age group PND 15. (8–12 injected cells were averaged for each animal). PND = postnatal day; scale bar = 100 μm .

Confocal analysis of dendritic spine development on second-order dendritic shafts of layer 5 mPFC pyramidal neurons revealed a nearly 10-fold increase in dendritic spine density between PND 5 and PND 30, and this effect was comparable between apical ($951 \pm 33\%$; $P < 0.001$) and basal ($894 \pm 90\%$; $P < 0.001$) dendritic segments (fig. 2). At PND 5, only a very small amount of dendritic protrusions were detectable and their number significantly increased by

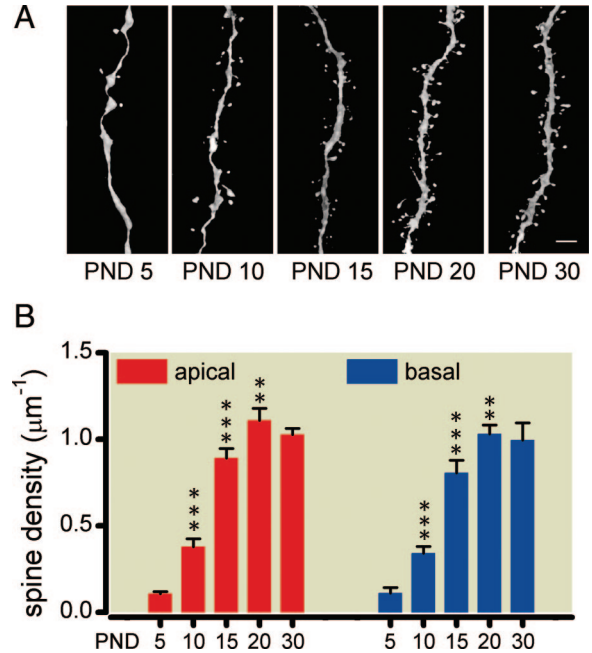


Fig. 2. Dendritic spine development of layer 5 pyramidal neurons in the medial prefrontal cortex of Wistar rats during the first month of postnatal life. (A) Representative confocal images (three-dimensional volume rendering) showing the temporal evolution of dendritic spine density on apical dendritic shafts of layer 5 pyramidal neurons between postnatal days 5 and 30. (B) Quantitative analysis of the temporal evolution of dendritic spine density on second order apical (red columns) and basal (blue columns) dendritic shafts during this period. Results are expressed as mean ± SD. ** = $P < 0.01$; *** = $P < 0.001$, one-way ANOVA with Bonferroni correction posttests performed between subsequent age groups for basal and apical dendritic spines. $n = 4$ animals per age group. A total of 3,557 spines for apical and 3,596 spines for basal dendrites were counted to determine spine density. PND = postnatal day; scale bar = 6 μm .

PND 10 on either apical (from 0.11 ± 0.01 to $0.38 \pm 0.05 \mu\text{m}^{-1}$; $P < 0.0001$) or basal (from 0.11 ± 0.03 to $0.34 \pm 0.04 \mu\text{m}^{-1}$; $P < 0.0001$) dendritic shafts. Further significant increase in protrusion density was found at PND 15 (apical: $0.89 \pm 0.06 \mu\text{m}^{-1}$, $P < 0.0001$; basal: $0.81 \pm 0.07 \mu\text{m}^{-1}$, $P < 0.0001$) and at PND 20 (apical: $1.1 \pm 0.07 \mu\text{m}^{-1}$, $P = 0.0026$; basal: $1.03 \pm 0.05 \mu\text{m}^{-1}$, $P = 0.0025$). In contrast, dendritic protrusion density remained unchanged at PND 30 (apical: $1.03 \pm 0.04 \mu\text{m}^{-1}$, $P = 0.076$; basal: $0.99 \pm 0.1 \mu\text{m}^{-1}$, $P = 0.55$), suggesting thereby that the most intense phase of spinogenesis is probably ceased by this developmental stage.

Propofol Anesthesia, Up to 6 h, Does Not Affect Dendritic Arbor Development of Layer 5 mPFC Pyramidal Neurons

Because neural activity plays a pivotal role in dendritic arbor development,³ we next evaluated the effect of anesthesia-induced interference with physiologic patterns of neural ac-

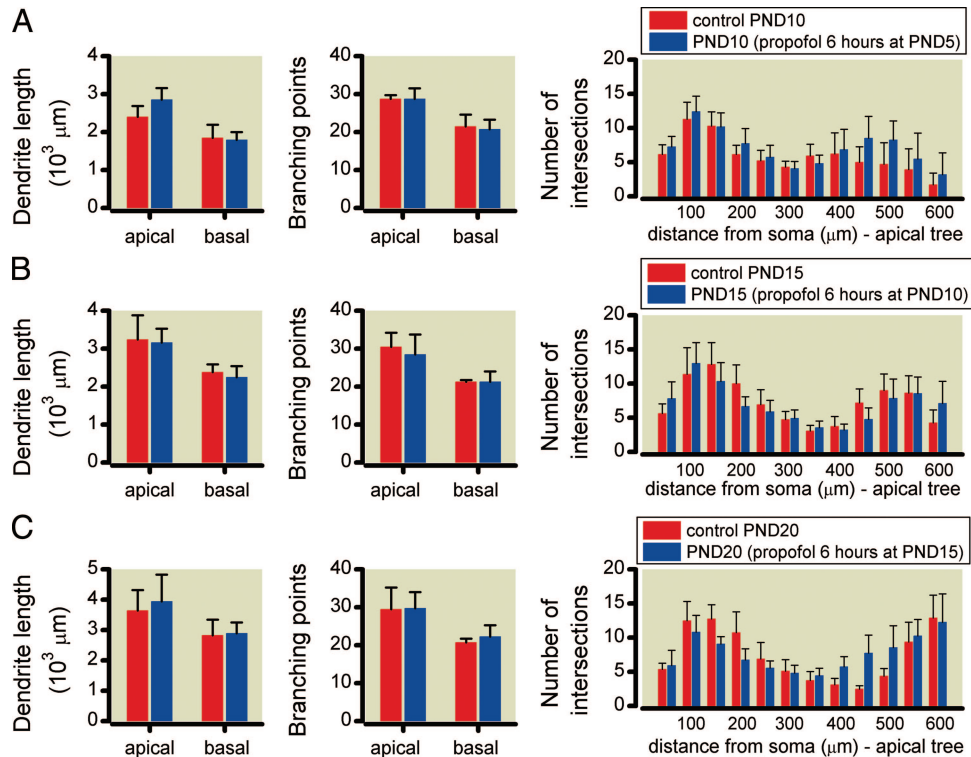


Fig. 3. Propofol anesthesia does not affect gross dendritic arbor development of layer 5 pyramidal neurons during the first month of postnatal life. Effects of a 6-h-long propofol anesthesia, performed at (A) postnatal day (PND) 5, (B) PND 10 or at (C) PND 15 on apical and basal dendritic length (left panels), branching points (middle panels) or three-dimensional Sholl distribution (right panels) as assessed 5 days after drug exposure (*i.e.*, at PND 10 for A; at PND 15 for B; and at PND 20 for C). Results are expressed as mean \pm SD. Two-tailed Student *t* test was used for searching statistical difference between control and propofol-exposed animals. All differences were found statistically nonsignificant. $n = 4$ animals per group of age for each condition (8–12 injected cells were averaged for each animal). PND = postnatal day.

tivity on the developing neuronal dendritic tree. To this aim, we conducted 6-h-long propofol anesthesia at PND 5, 10, or 15, and evaluated the effects of these treatment paradigms on dendritic arbor architecture of layer 5 mPFC pyramidal neurons 5 days after drug administration. Because apical and basal dendrites develop differently,^{22,23} we discriminated between these two entities throughout the entire study. Exposure to propofol up to 6 h at any of these aforementioned developmental stages had no effect on dendritic length, the number of branching points, or three-dimensional spatial distribution of apical (fig. 3) or basal (not shown) dendritic tree.

Developmental Stage- and Exposure Time-dependent Acute Effects of Propofol Anesthesia on Dendritic Spine Density and Shape

To further investigate the impact of propofol on neuronal circuitry development, we then systematically evaluated the effects of this drug, 6 h after the initiation of anesthesia, on dendritic spine density at distinct developmental stages, using either a single dose or a 6-h-long exposure time period. The ensemble of these investigations revealed that the effect of propofol on dendritic spines was dependent on the developmental stage at which this drug was administered (fig. 4). At PND 5, after administration of a single dose of propofol

(50 mg/kg intraperitoneal), there was a $47 \pm 20\%$ decrease in dendritic spine density on apical (from 0.11 ± 0.01 in control to $0.06 \pm 0.02 \mu\text{m}^{-1}$ in propofol group; $P = 0.0067$), and a $68 \pm 19\%$ decrease (from 0.11 ± 0.03 to $0.04 \pm 0.02 \mu\text{m}^{-1}$; $P = 0.0065$) on basal dendritic shafts of layer 5 pyramidal neurons in the mPFC. When animals were exposed to a 6-h-long propofol anesthesia at this developmental stage, the decrease in spine density reached $84 \pm 7\%$ ($P < 0.0001$) on apical, and $76 \pm 11\%$ ($P = 0.0022$) on basal dendritic segments. Importantly, the difference between the single dose and the 6-h-long propofol exposure reached statistical significance on apical ($P = 0.0134$) but not on basal shafts ($P = 0.49$) of these neurons.

Exposure to propofol at PND 10 also induced a significant decrease in dendritic spine density, although the extent of decrease was less pronounced than at PND 5 (fig. 4). On apical dendritic shafts, we observed a $29 \pm 6\%$ decrease in spine density after administration of a single dose of propofol (from 0.38 ± 0.05 in control to $0.27 \pm 0.02 \mu\text{m}^{-1}$ in the propofol group; $P = 0.0065$), and a rather similar extent of decrease was found after a 6-h-long exposure to this drug ($-27 \pm 5\%$; $0.27 \pm 0.02 \mu\text{m}^{-1}$; $P = 0.0069$). On basal dendrites, single-dose propofol injection decreased spine density by $36 \pm 12\%$ (from 0.34 ± 0.04 to 0.22 ± 0.04

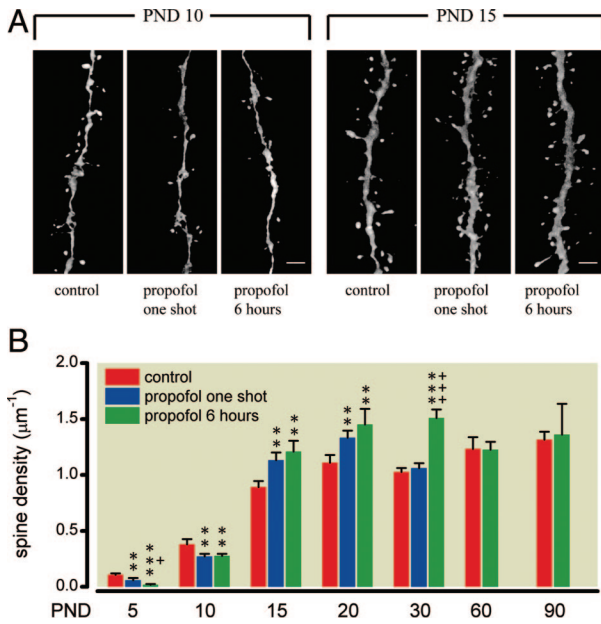


Fig. 4. Developmental stage-dependent early effects of propofol anesthesia on dendritic spines. (A) Representative confocal images (three-dimensional volume rendering) showing the early effects of a single dose as well as a 6-h-long propofol treatment on apical dendritic spine density at postnatal day 10 (left panel) and 15 (right panel). (B) Quantitative analysis showing the developmental stage-dependent effects of a single bolus and a 6-h-long propofol treatment on dendritic spine density on apical dendrites. Results are expressed as mean \pm SD. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$, one-way ANOVA with Bonferroni correction posttests within each group of age for control *versus* propofol one shot or 6-h treatment; + = $P < 0.05$; +++ = $P < 0.001$, one-way ANOVA with Bonferroni correction posttests within each group of age for propofol one shot *versus* propofol 6-h treatment. $n = 4$ animals per age group for each condition. In each age group, controls are identical to those shown originally in figure 2. A total of 6,327 spines for controls, 3,773 spines for single-bolus propofol, and 5,452 spines for the 6-h-long treatment were counted to determine spine density. PND = postnatal day; scale bar = 5 μm .

μm^{-1} ; $P = 0.0059$), whereas the 6-h-long treatment induced a comparable 33 \pm 8% reduction (to 0.23 \pm 0.03 μm^{-1} ; $P = 0.0037$).

In contrast to these aforementioned earlier time points, we found a significant increase in dendritic spine density when this drug was administered at PND 15 (fig. 4). At this developmental stage, on apical shafts, single-dose administration of propofol increased spine density by 27 \pm 7% (from 0.89 \pm 0.06 to 1.13 \pm 0.07 μm^{-1} ; $P = 0.0015$), and there was a proportionate 36 \pm 11% increase (to 1.21 \pm 0.09 μm^{-1} ; $P = 0.0013$) after the 6-h-long treatment with this drug. On basal dendrites, we also found a comparable increase in spine density either after the single dose (+19 \pm 5%, from 0.81 \pm 0.07 to 0.97 \pm 0.04 μm^{-1} ; $P = 0.013$) or after the 6-h-long (+28 \pm 7%, to 1.03 \pm 0.06 μm^{-1} ; $P = 0.0029$) propofol exposure.

At PND 20 (fig. 4), propofol also induced a significant increase in spine density on apical dendrites after a single dose (+20 \pm 6%; from 1.1 \pm 0.07 to 1.33 \pm 0.06 μm^{-1} ; $P = 0.003$) as well as after a 6-h-long treatment (+31 \pm 12%; to 1.45 \pm 0.07 μm^{-1} ; $P = 0.0045$). In contrast, on basal dendrites, no significant increase in spine density was found after a single-bolus propofol injection (+11 \pm 9% from 1.03 \pm 0.05 to 1.14 \pm 0.09 μm^{-1} ; $P = 0.083$) whereas the 6-h-long treatment paradigm still produced a significant effect (+20 \pm 6%, to 1.24 \pm 0.06 μm^{-1} ; $P = 0.0021$).

At PND 30, we found no significant difference in dendritic spine density after single-dose propofol exposure either on apical (+3 \pm 4% from 1.03 \pm 0.04 to 1.06 \pm 0.04 μm^{-1} ; $P = 0.28$) or on basal (-1 \pm 7% from 0.99 \pm 0.1 to 0.89 \pm 0.07 μm^{-1} ; $P = 0.12$) dendritic shafts (fig. 4). However, the 6-h-long exposure induced a highly significant increase in spine density on the whole dendritic tree (apical: +47 \pm 7% to 1.5 \pm 0.08 μm^{-1} , $P < 0.0001$; basal: +33 \pm 9% to 1.32 \pm 0.09 μm^{-1} , $P = 0.0031$).

To investigate whether propofol anesthesia also modifies dendritic spine density at later stages of postnatal life, we exposed male rats to this drug at PND 60 and 90. Results of these experiments revealed that up to a 6-h-long exposure to propofol affects dendritic spine density at these stages neither on apical (at PND 60: -1 \pm 5% from 1.23 \pm 0.1 to 1.23 \pm 0.07 μm^{-1} , $P = 0.91$; at PND 90: +3 \pm 20% from 1.32 \pm 0.07 to 1.36 \pm 0.28 μm^{-1} , $P = 0.76$) nor on basal dendritic shafts (at PND 60: -0.5 \pm 2% from 1.05 \pm 0.09 to 1.04 \pm 0.03 μm^{-1} , $P = 0.94$; at PND 90: +3 \pm 14% from 1.19 \pm 0.08 to 1.22 \pm 0.17 μm^{-1} , $P = 0.72$), indicating that these effects are most probably restricted to critical periods of early postnatal life (fig. 4).

The Propofol-induced Changes in Dendritic Spine Density Correlate with a Selective Decrease or Increase of Specific Spine Populations

In order to obtain additional insight into how propofol anesthesia may modulate dendritic spine development, we also assessed the developmental stage-dependent effects of this drug on the morphology of dendritic spine head, because this parameter has been previously shown to be correlated with synaptic strength.^{24,25} As an approximation of the estimation of spine volume, we measured the maximal cross sectional diameter of the protrusion head as determined by analysis of confocal image stacks (see Materials and Methods). Analysis using this technology revealed that the propofol-induced decrease in spine density, observed at PND 5 and at PND 10 on both apical and basal dendritic shafts, is primarily caused by a significant decrease in the number of spines with a head diameter between 0.3 and 0.4 μm , and to a lesser, nonsignificant extent, to the decrease of spines with head diameters larger than 0.5 μm (fig. 5A). In contrast, the propofol-induced increase in spine density, observed between PND 15 and PND 30, was primarily

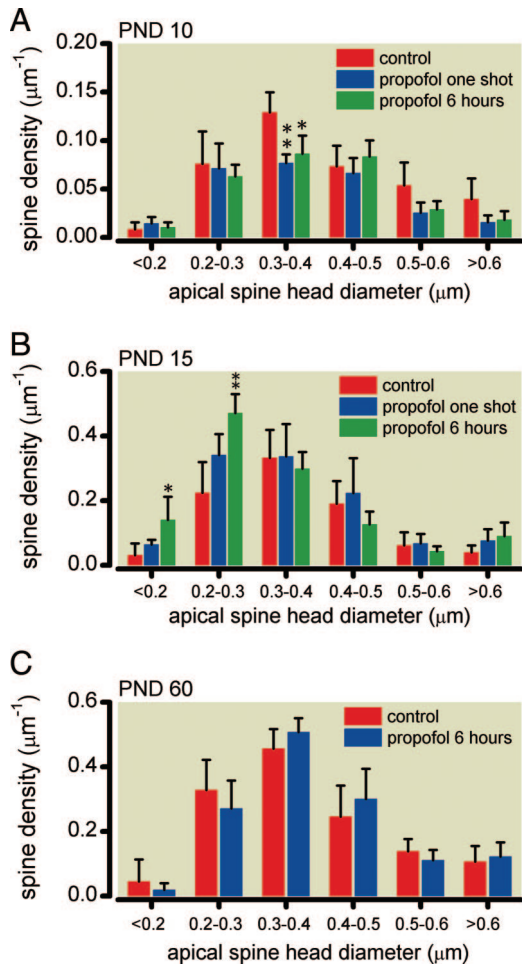


Fig. 5. Early effects of propofol anesthesia on dendritic spine morphology. Frequency distribution histograms of apical dendritic spine head diameters in control (red columns), single-bolus propofol (blue columns) and 6-h-long propofol exposure (green columns) groups showing the early effects of these treatment paradigms on dendritic spine morphology at postnatal days 10 (A), 15 (B), and 60 (C). Results are expressed as mean \pm SD. * = $P < 0.05$; ** = $P < 0.01$; one-way ANOVA with Bonferroni correction posttests within each group of diameter for control versus propofol one shot or 6 h treatment. $n = 4$ animals per experimental group for each developmental stage (8–12 injected cells were averaged for each animal, at total 710 spines were analyzed in A, 792 in B, and 499 in C). PND = postnatal day.

caused by an increase in the number of spines with a head diameter of less than $0.3 \mu\text{m}$ (fig. 5B). Finally, propofol anesthesia up to 6 h long did not seem to influence dendritic spine morphology at PND 60 and at PND 90 (fig. 5C), further suggesting that the effect of this drug on the morphology of neural circuitry is limited to critical periods of development.

Propofol-induced Increase in Dendritic Spine Density is Accompanied by an Increase in Spine Synapse Number

Although dendritic spines are widely considered to represent excitatory synaptic contacts, an important aspect was to ascertain whether the propofol-induced changes in dendritic

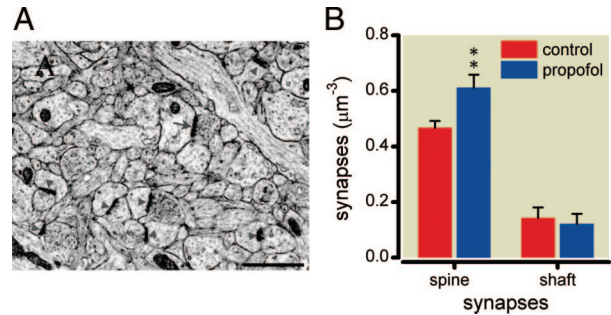


Fig. 6. Electron microscopy analysis of synaptic density 24 h after a 5 h-propofol anesthesia. (A) Electron microscopy illustration of the prefrontal cortex neuropil of PND 16 rats. Spine synapses are marked with red arrowheads, and shaft synapse is identified with a gray arrow. Scale bar = $1 \mu\text{m}$. (B) Spine and shaft synapse density (μm^{-3}) in control animals and 24 h after a 6-h-long propofol anesthesia. Results are expressed as mean \pm SD. ** = $P < 0.01$, two-tailed Student t test, $n = 3$ animals per condition.

spine density also represent changes in synapse number. Because previous studies revealed decreased synaptic density after anesthesia exposure at early postnatal stages (PND 0 to PND 7),^{16,17} here we assessed whether the propofol-induced increase in dendritic spine density that we observed at later postnatal stages correlates with an increased synaptic density. To this aim, we used an electron microscopy approach and the disector method to quantify the density of synapses in PND 15 rats exposed to propofol for 6 h and sacrificed 24 h later. Figure 6 illustrates the density of synapses observed in the PND 16 cerebral cortex after propofol anesthesia. Quantification of the number of synapses per volume after propofol treatment shows a significant $31 \pm 7\%$ increase in the number of spine synapses (from 0.47 ± 0.02 in control animals to $0.61 \pm 0.035 \mu\text{m}^{-3}$ in propofol-exposed animals; $P < 0.01$) whereas the number of shaft synapses remained comparable to that found in nonanesthetized control animals (0.14 ± 0.03 vs. $0.12 \pm 0.03 \mu\text{m}^{-3}$ in control and propofol-exposed animals, respectively; $P = 0.23$). These results strongly suggest that propofol anesthesia at PND 15 specifically induces an increase in the number of spine synapses.

Propofol Exposure in the Early Postnatal Period Induces Persistent Changes in Dendritic Spine Density and Morphology in the Medial Prefrontal Cortex

An important next step was to determine whether the propofol anesthesia-induced acute changes in dendritic spine density persist over time. We performed propofol anesthesia (single bolus injection and a 6-h-long exposure) either at PND 5 or at PND 15, and evaluated the effects of these paradigms on dendritic spine density 5 days after drug treatment (*i.e.*, at PND 10 and at PND 20, respectively) as well as in adult animals (PND 90).

Either single-bolus or a 6-h-long propofol anesthesia, performed at PND 5, induced a significant decrease in dendritic

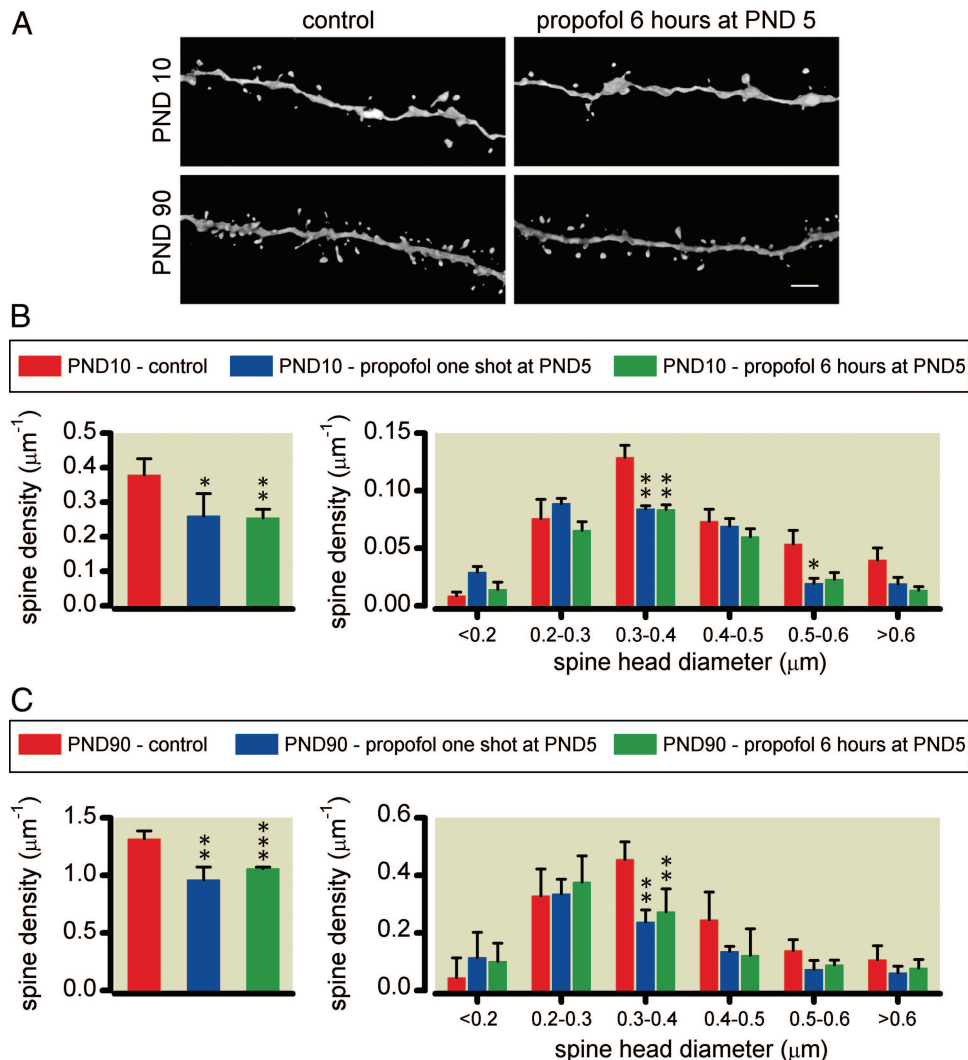


Fig. 7. Propofol anesthesia at postnatal day 5 induces persistent decrease in dendritic spine density. (A) Representative confocal images (three-dimensional volume rendering) showing the effects of propofol anesthesia, performed at postnatal day (PND) 5, on dendritic spines at PND 10 and 90. (B and C) Quantitative analysis of spine density (left panels) and spine head diameters (right panels) at PND 10 (B) and at PND 90 (C) reveals a significant difference between control animals (red columns) and those who received a single bolus (blue columns) or a 6-h-long propofol anesthesia (green columns) at PND 5. Results are expressed as mean \pm SD. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$, one-way ANOVA with Bonferroni correction posttests. At PND 10 and 90, controls are identical to those shown originally in Figs. 2 and 4. Left panels: both one-shot and 6-h propofol treatment groups are compared with the control group. Right panels: the same comparisons are done inside each diameter range classes. $n = 5$ animals per age group for each condition (8–10 neurons per animal), representing a total of 3,294 spines in B, left, and 3,563 spines in C, left, 756 spine heads in B, right, and 832 spine heads in C, right. PND= postnatal day; scale bar = 5 μm .

spine density on apical (single bolus: $-31 \pm 17\%$, from 0.38 ± 0.05 to $0.26 \pm 0.06 \mu\text{m}^{-1}$, $P = 0.026$; 6-h-long: $-33 \pm 7\%$ to $0.25 \pm 0.03 \mu\text{m}^{-1}$, $P = 0.0036$) as well as on basal (single bolus: $-44 \pm 13\%$, from 0.34 ± 0.04 to $0.22 \pm 0.04 \mu\text{m}^{-1}$, $P = 0.0079$; 6-h-long: $-36 \pm 8\%$, to $0.23 \pm 0.03 \mu\text{m}^{-1}$, $P = 0.0036$) dendrites when evaluated at PND 10 (fig. 7A–B). Most importantly, evaluation of dendritic spines at PND 90 in male rats who underwent propofol anesthesia at PND 5 still revealed highly significant reductions in spine density in comparison with non-anesthetized control animals of the same age and sex (figs. 7A and C). On apical dendrites, we found a $27 \pm 8\%$

decrease (from 1.32 ± 0.07 to $0.96 \pm 0.11 \mu\text{m}^{-1}$, $P = 0.0017$) after a single bolus, and a $20 \pm 1\%$ drop (to $1.06 \pm 0.01 \mu\text{m}^{-1}$, $P = 0.0004$) after the 6-h-long propofol administration. Comparably, on basal dendrites, there was a $24 \pm 10\%$ decrease (from 1.19 ± 0.07 to $0.9 \pm 0.12 \mu\text{m}^{-1}$, $P = 0.0067$) in the single dose, and a $14 \pm 6\%$ decrease (to $1.02 \pm 0.08 \mu\text{m}^{-1}$, $P = 0.019$) in the 6-h-long group. Analysis of dendritic spine head diameter, either at PND 10 or at PND 90, revealed that the decrease in spine density is primarily linked to the selective reduction in the number of spines with a head diameter between 0.3 and 0.4 μm (fig. 7).

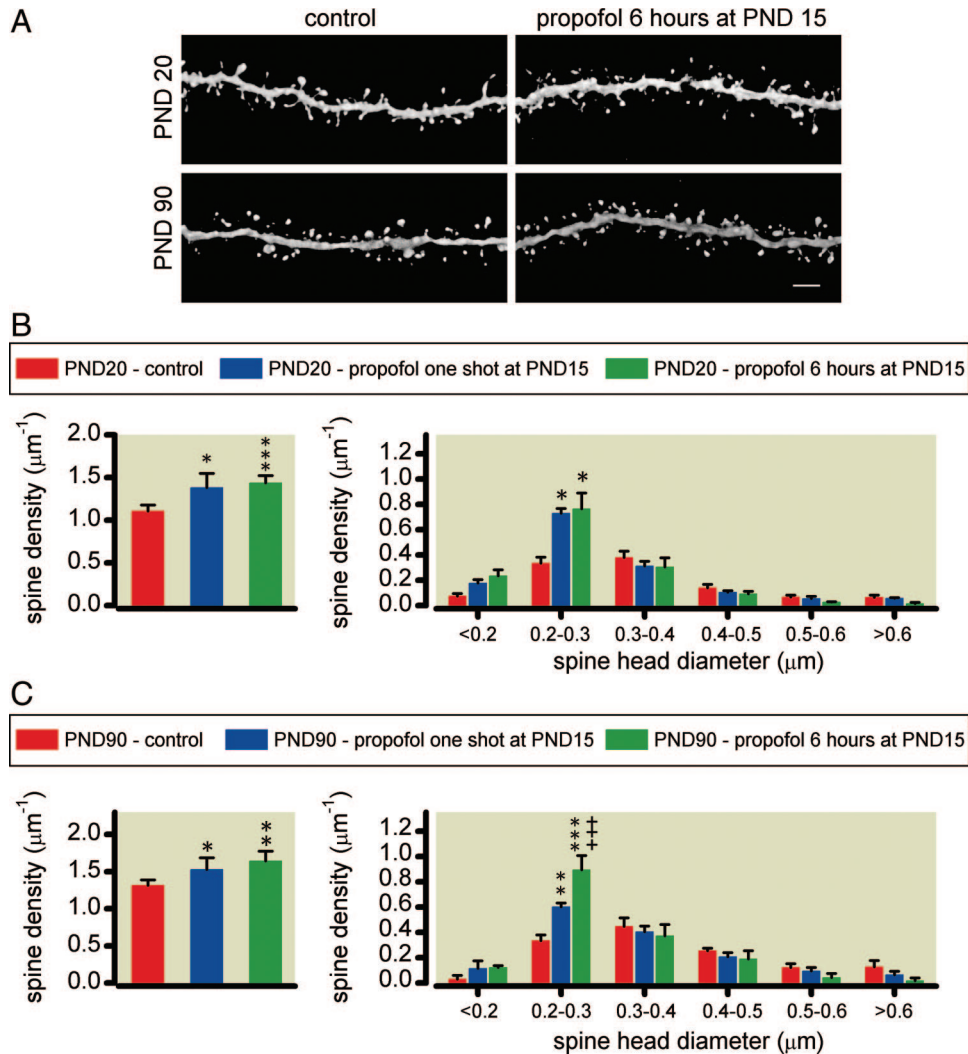


Fig. 8. Propofol anesthesia at postnatal day (PND) 15 induces persistent increase in dendritic spine density. (A) Representative confocal images (three-dimensional volume rendering) showing the effects of propofol anesthesia, performed at PND 15, on dendritic spines at PND 20 and 90. (B and C) Quantitative analysis of spine density (left panels) and spine head diameters (right panels) at PND 10 (B) and at PND 90 (C) reveals a significant difference between control animals (red columns) and those who received a single bolus (blue columns) or a 6-h-long propofol anesthesia (green columns) at PND 15. Results are expressed as mean \pm SD. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$, one-way ANOVA with Bonferroni correction posttests. At PND 10 and 90, control animals are identical to those shown originally in Figs. 2 and 4. Left Panels: both one-shot and 6-h propofol treatment groups are compared with the control group. Right panels: same comparisons but inside each diameter range classes. +++ = $P < 0.001$, one-way ANOVA with Bonferroni correction posttests comparing single-bolus propofol versus 6-h propofol group's spine density in within the same head diameter range class. $n = 5$ animals per age group for each condition (8–10 neurons per animal), representing a total of 4,429 spines in B, left, 4,143 spines in C, left, 870 spine heads in B, right, and 10,006 heads in C, right. PND = postnatal day; scale bar = 5 μm .

Administration of propofol at PND 15 also induced significant lasting changes in dendritic spines. Five days after single-bolus propofol anesthesia, we found a $25 \pm 15\%$ increase (from 1.1 ± 0.07 to $1.38 \pm 0.17 \mu\text{m}^{-1}$, $P = 0.022$) in apical and a $26 \pm 9\%$ increase (from 1.03 ± 0.05 to $1.30 \pm 0.09 \mu\text{m}^{-1}$, $P = 0.0022$) in basal dendrites compared with control, sham-treated groups of the same age (fig. 8A–B). A comparable increase in spine density was found in the 6-h-long group either on apical ($+30 \pm 8\%$, to $1.44 \pm 0.08 \mu\text{m}^{-1}$, $P = 0.0009$) or on basal ($+43 \pm 19\%$, to $1.47 \pm 0.07 \mu\text{m}^{-1}$, $P = 0.0042$) dendritic shafts. Analysis at

PND 90 still revealed significant difference between control animals and those that received a single bolus of propofol anesthesia at PND 15 either on apical ($+16 \pm 12\%$, from 1.32 ± 0.07 to $1.5 \pm 0.02 \mu\text{m}^{-1}$, $P = 0.046$) or on basal ($+39 \pm 5\%$, from 1.17 ± 0.06 to $1.65 \pm 0.06 \mu\text{m}^{-1}$, $P = 0.44$) dendrites (fig. 8A, C). The 6-h-long propofol exposure at this same developmental stage also induced significant lasting changes in spine density both on apical ($+25 \pm 10\%$, to $1.64 \pm 0.13 \mu\text{m}^{-1}$, $P < 0.0001$) and on basal ($+13 \pm 5\%$, to $1.35 \pm 0.05 \mu\text{m}^{-1}$, $P = 0.0204$) shafts. Either at PND 20 or at PND 90, the observed lasting increase in

dendritic spine density was associated with an important increase in the number of dendritic spines with a head diameter of less than $0.3 \mu\text{m}$ (fig. 8).

Discussion

Here we provide *in vivo* morphologic evidence that even a single-bolus exposure to the general anesthetics propofol can rapidly induce enduring structural modifications of developing mPFC neural circuitry in a developmental stage-dependent manner during cortical synaptogenesis. By focusing on dendritic arbor development of layer 5 mPFC pyramidal neurons in rat pups, we first demonstrate that these cells undergo intense differentiation and synaptogenesis during the first postnatal month. Next, by systematically evaluating the effects of propofol administration at defined developmental time points between PND 5 and 30, we show that up to a 6-h-long exposure to this drug does not interfere with gross dendritic arbor development of these cells. In contrast, even single-bolus administration of propofol is sufficient to rapidly induce substantial modifications in dendritic spines, representing primary sites of excitatory synaptic contacts in these neurons. Importantly, this effect is developmental stage-dependent, because propofol decreases spine density when administered to rat pups until PND 10 but increases the number of these synaptic contacts when animals are exposed between PND 15 and 30. Finally, long-term follow-up experiments revealed that the propofol-induced rapid changes in dendritic spine density during the early postnatal period persist into adulthood. These results thus strongly suggest that even short-term exposure to propofol during critical periods of brain development might permanently alter neural connectivity. They also point to the fact that this drug might affect circuitry development rather differently depending on the developmental stage at which it is administered.

To our knowledge, this is the first study designed to systematically evaluate the effects of propofol at distinct developmental stages extending through the entire time span of cortical synaptogenesis. Recent studies demonstrated an anesthetics-induced decrease in synapse densities.^{16,17} In contrast, we have previously provided evidence that these very same drugs can also induce synaptogenesis.^{18,19} The major difference between our and these other studies was the developmental stage at which animals were exposed to anesthetics. Either isoflurane alone¹⁶ or a mixture of midazolam/isoflurane/nitrous oxide anesthesia¹⁷ decreased synaptic density when pups were exposed to these drugs at PND 5 or 7. In contrast, our studies showed that either intravenous or volatile anesthetics increased synaptic density in animals older than 2 weeks. We thus believe that data provided in the current study conciliate these seemingly opposite observations, and strongly suggest a developmental stage-dependent, multifaceted role of propofol and potentially other general anesthetics on synaptogenesis.

The molecular mechanisms underlying these developmental stage-dependent differences of propofol on synap-

genesis remain to be elucidated. One plausible explanation would concern the differential modulation of excitation/inhibition activity balance by this drug at early *versus* later stages of development. GABAergic neurotransmission is initially excitatory and the functional transition toward inhibitory modalities occurs during the second postnatal week in the rodent cerebral cortex.^{26–28} An alternative or concomitant possibility is that the developmental stage-dependent effects of anesthetics on circuitry development are related to the important changes in GABA_A receptor subunit composition during early postnatal life^{29–31} which, in turn, significantly modify GABAergic inhibitory tone modalities.³² In light of some recent observations,¹⁶ a developmental stage-dependent differential effect of propofol on neurotrophin factor signaling cannot be excluded either. Clearly, additional studies are needed to answer these questions.

Our data strongly suggest that even brief exposure to propofol during critical periods of early postnatal life can rapidly alter the morphology of developing brain circuitry. These results are based on *post hoc ex vivo* imaging of dendritic arbor after iontophoretic injection of Lucifer Yellow into layer 5 pyramidal neurons of the mPFC. This method is extensively validated and allows complete visualization of gross dendritic arbor as well as dendritic spines,^{33,34} with dendritic spines representing postsynaptic sites of excitatory synaptic contacts onto these neurons.²⁴ Using this approach, we have shown a more than 10-fold increase in the number of dendritic spines during the first postnatal month. These results are in agreement with electron microscopy data describing cortical synaptogenesis during the same time period,¹³ further confirming the validity of this technique to follow synaptogenesis. Importantly, this assumption is corroborated with our own observations demonstrating that the propofol-induced rapid increase in dendritic spine density is accompanied by a significant increase of excitatory spine synapses, as revealed by electron microscopy. Because general anesthetics are known to affect neurotrophin signaling¹⁶ and that significant differences has been observed between apical and basal pyramidal dendrites in terms of neurotrophin factor responsiveness,^{22,23} we focused on both apical and basal dendritic arbor. The fact that the single-bolus propofol administration rapidly induced significant and rather similar amount of changes in either apical or in basal dendritic spine density is in accord with our recent observations demonstrating that volatile anesthetics can rapidly induce both apical and basal dendritic spinogenesis in the mPFC,¹⁹ and suggests that those anesthetics act as potentiators of the GABA_A receptor might all exert rather similar effects on synaptogenesis.

One important observation of our current work is that the propofol-induced developmental stage-dependent changes in dendritic spine densities during the early postnatal period persisted up to 3 months of age, representing the adult stage in rodents. To our knowledge, this is the first demonstration that brief pharmacologic interference with excitation/inhibition activity balance during distinct stages of the early post-

natal period can result in permanent morphologic alterations of neural circuitry. Earlier studies, assessing the long-term effects of halothane on brain development, revealed that this volatile anesthetic, when chronically administered at low levels either *in utero* or during the first months of postnatal life, induces permanent decrease in synaptic density and also partially destroys neuropil.^{35–39} More recently, a 6-h-long midazolam/isoflurane/nitrous oxide anesthesia in PND 7 rat pups has been reported not only to reduce synapse density but also to impair neuropil structure in the subiculum, and the effect persisted at least up to 2 weeks after exposure.¹⁷

In addition to alterations in dendritic spine density, morphologic assessment of dendritic spine shape also revealed important and lasting differences between control and propofol-exposed animals. Because dendritic spines play a key role in the expression of synaptic plasticity,⁴⁰ these observations further underscore the potential of anesthetics to interfere with neural development and function. Indeed, several potential links between spine morphology and function have been reported.²⁴ Among them, spine head volume has been shown to be directly proportional to the size of post-synaptic density, the presynaptic number of docked vesicles, and thus the readily releasable pool of neurotransmitters.^{41–44} As an estimation of the spine head volume, here we measured spine head diameter and showed that the propofol-induced decrease in protrusion density is associated with a concomitant reduction in the number of middle- to large-head-bearing, presumably more mature dendritic spines. In contrast, the propofol-induced increase in spine density was accompanied by a selective increase of dendritic spines with small head diameters representing, most probably, immature newly generated spines. An intriguing observation was that these morphologic alterations persisted into adulthood, raising the possibility that dendritic spine dynamics could be lastingly altered.⁴⁵ In line with this possibility, using organotypic hippocampal slices, we have recently shown that midazolam-induced increase in spine density is mediated through an increased rate of protrusion formation, a better stabilization of newly formed spines that, ultimately, led to an increased formation of functional synapses.¹⁸ Future experiments should target whether and how developmental stage-dependent anesthesia exposure might alter dendritic spine dynamics and thereby synaptic plasticity *in vivo*. Currently, because *in vivo* tracing of dendritic spine plasticity is only feasible under general anesthesia, these experiments would be hindered by the necessity to expose the animals to anesthetics.

Extrapolation of the developmental stages between rodent and human development is a difficult issue. In this study, we focused on the entire time span of rodent synaptogenesis, an approximately 3-week-long period that takes place between the second and fourth week of postnatal life.^{11–13} The synaptogenic period is much longer on the human scale, starting during the third trimester of pregnancy and extending up to the first few years of postnatal life.^{7–9} For example, a continuously growing number of synaptic

contacts up to 4 yr of age have been reported in the human prefrontal cortex.¹⁰ Recent studies, attempting to draw temporal correlation between rodent and human brain development, suggest that the developmental stage of rodent brains at PND 5–7 corresponds to the maturational state of the human brain at the very beginning of the third trimester of pregnancy, whereas the period between PND 15 and 20 might reflect a period somewhere during the first few years at the human scale.^{46,47} Our study is in line with these assumptions because we show that significant dendritic spinogenesis takes place between PND 5 and 20. This relatively long time span of synaptogenesis together with our results showing developmental stage-dependent differential effects of propofol on dendritic spines clearly show that the effects of anesthetics on neural network development are multifaceted and cannot be evaluated by focalizing on one defined developmental stage.

In conclusion, this study demonstrates that propofol anesthesia induces developmental stage-dependent persistent morphofunctional alterations in developing mPFC neural circuitry. These results complete our recent series of data demonstrating that anesthetics can rapidly induce synaptogenesis during critical periods of brain development,^{18,19} and further strengthen the view that these drugs can act as powerful modulators of synaptic plasticity during critical periods of brain development.

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