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Research Article

Acute and Long-Term Effects of Brief Sevoflurane Anesthesia During the Early Postnatal Period in Rats

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ABSTRACT

The possibility that exposure to general anesthetics during early life results in long-term impairment of neural function attracted considerable interest over the past decade. Extensive laboratory data suggest that administration of these drugs during critical stages of central nervous system development can lead to cell death, impaired neurogenesis, and synaptic growth as well as cognitive deficits. These observations are corroborated by several recent human epidemiological studies arguing that such cognitive impairment might also occur in humans. Despite the potential public health importance of this issue, several important questions remain open. Amongst them, how the duration of anesthesia exposure impact on outcome is as yet not fully elucidated. To gain insight into this question, here we focused on the short- and long-term impact of a 30-min-long exposure to clinically relevant concentrations of sevoflurane in rat pups at 2 functionally distinct stages of the brain growth spurt. We show that this treatment paradigm induced developmental stage-dependent and brain region-specific acute but not lasting changes in dendritic spine densities. Electrophysiological recordings in hippocampal brain slices from adult animals exposed to anesthesia in the early postnatal period revealed larger paired-pulse facilitation but no changes in the long-term potentiation paradigm when compared with nonanesthetized controls. 5-bromo-2-deoxyuridine pulse and pulse-chase experiments demonstrated that neither proliferation nor differentiation and survival of hippocampal progenitors were affected by sevoflurane exposure. In addition, behavioral testing of short- and long-term memory showed no differences between control and sevoflurane-exposed animals. Overall, these results suggest that brief sevoflurane exposure during critical periods of early postnatal development, although it does not seem to exert major

long-term effects on brain circuitry development, can induce subtle changes in synaptic plasticity and spine density of which the physiological significance remains to be determined.

Key words: anesthesia; brain; neurodevelopment; neurogenesis; neurotoxicity; memory

The possibility that exposure to anesthesia during early life might induce long-term adverse effects on the central nervous system (CNS) is a current global concern with potential public health implications (Rappaport *et al.*, 2015; Servick, 2014). Laboratory observations in rodents along with more recent experimental data in nonhuman primates show that exposure to general anesthetics during the early postnatal period can induce cell death, impaired synapse growth and neurogenesis as well as subsequent cognitive and behavioral problems (Jevtovic-Todorovic, 2013; Jevtovic-Todorovic *et al.*, 2003; Lin *et al.*, 2014). Although several retrospective cohort studies indicate a possible association between early childhood exposure to anesthesia and surgery and subsequently altered neurocognitive performance, there is conflicting information as to whether this is a general public health issue or rather related to certain vulnerable patient groups (Lei *et al.*, 2014). Despite the available laboratory and clinical data, many important questions remain unanswered (Vutskits *et al.*, 2012). From a clinical perspective, prospective randomized trials to investigate neurological adverse outcomes after early childhood anesthesia and surgery are warranted. As for laboratory work, the translational relevance of experimental models and designs remains a critical issue.

Given the important ethical, financial and technical constraints regarding experimentation in nonhuman primates, rodent models remain the mainstay of laboratory investigations in the field of developmental anesthesia neurotoxicity. Yet, several issues should be taken into account when considering the translational relevance of works in rodent species. One of them is that while in humans the brain growth spurt extends from the last trimester of pregnancy up to the first few years of life, this same period is limited to a little <1 month in rodents (Clancy *et al.*, 2007; Semple *et al.*, 2013; Vutskits, 2012). This implies that the physiological relevance of a given length of drug exposure might be fundamentally different between rodents and humans. It is moreover difficult to draw definite conclusions on drug effects on the developing brain by focusing on 1 single time point of exposure. Indeed, signaling through ion channels and growth factor receptors can be fundamentally different between earlier and later stages of the brain growth spurt meaning that anesthetics can even have opposite effects when administered at 2 distinct time points of early postnatal development (Semple *et al.*, 2013; Vutskits, 2012).

Sevoflurane is amongst the most commonly used anesthetics in pediatric practice with typically short exposure durations for both diagnostic and interventional procedures in neonates and children (Goa *et al.*, 1999). Several recent experimental works, from rodents to nonhuman primates, suggest that exposure to this anesthetic during early postnatal life can induce lasting impairment of CNS microarchitecture and function (Amrock *et al.*, 2015; Chung *et al.*, 2015; Liu *et al.*, 2015; Makaryus *et al.*, 2015; Shen *et al.*, 2013b). The majority of these investigations included several-hour-long exposures and little is known about whether and how shorter exposure times can also affect the assembly and function of neuronal networks. Hence, this study was aimed at providing detailed morphological as well as functional evaluation of the impact of a brief sevoflurane exposure during the brain growth spurt. We exposed neonatal rat pups to clinically relevant concentrations of sevoflurane (2.5%) for 30 min at 2 functionally distinct stages of the brain growth spurt and evaluated both the acute and long-term effects of this treatment paradigm on a large palette of morpho-functional parameters, including dendritic spinogenesis and circuitry function as well as neurogenesis and long-term cognitive performance.

MATERIALS AND METHODS

Animals

A multicenter trial design was implemented with investigators from Stockholm (Karolinska Institutet and Karolinska University Hospital), Gothenburg (University of Gothenburg), and Geneva (University Medical Center) performing the same consented protocol. The respective Ethics Committees at each Institution approved the study and efforts were made to minimize the number of animals used and their suffering. Male Wistar rats, 7-day old (PD7) and 15-day old (PD15), were used in the study. All animals were housed in a temperature- and humidity-controlled environment with a 12-h light-dark cycle and fed standard rodent chow and water *ad libitum*.

Anesthesia

Animals (PD7 and 15) were placed awake in a chamber for 30 min to be exposed to either air as a control for the maternal separation or sevoflurane anesthesia at 2.5% inspired fraction. Sevoflurane (Baxter) anesthesia was induced at 5% until loss of righting reflex was achieved and maintained at 2.5% in 0.30 FiO_2. Chamber concentrations of carbon dioxide, oxygen, and sevoflurane were constantly monitored (Capnomac Ultima; Datex-Ohmeda, Helsinki, Finland) while arterial pulse oximetry was continuously recorded (Kent Scientific, Torrington, Connecticut) and pups visually inspected every 5 min for any distress. Body temperature was monitored throughout the exposure using a homoeothermic pad system (Kent Scientific) as verified with a rectal probe at 36.5°C ± 0.5°C. After anesthesia, pups were allowed to recover in a warm environment until righting reflex was regained. Pups were then smeared with bedding from their home cage and returned to the dam until weaning at PD21.

Iontophoretic Post Hoc Single Cell Injections and Dendritic Spine Density Analysis

At defined experimental time points, animals were sacrificed by an overdose of pentobarbital (100 mg/kg intraperitoneally) and perfused transcardially with a 4% paraformaldehyde (PFA), 0.125% glutaraldehyde (pH 7.4) solution. Brains were then removed and postfixed for 2 h in 4% PFA as described in Briner *et al.*, (2010). Briefly, 300 μm thick coronal sections of the left hemisphere were cut on a vibratome in ice-cold phosphate-based saline solution (PBS, pH 7.4). Coronal sections were prestained for 30 min with methylene blue which enabled the visualization of neuronal somata, mounted into an injection chamber, and placed on a fixed stage Zeiss microscope

equipped with a micromanipulator. Layer 2/3 pyramidal neurons in the medial prefrontal and somatosensory cortices as well as CA1 pyramidal neurons in the hippocampus were loaded iontophoretically with a 5% Lucifer Yellow solution (Sigma-Aldrich, St Louis, Missouri) using sharp micropipettes with a negative current of 70 nA. Loading time per cell was 4 min. Six to nine cells were loaded per animal.

For immunolabeling to detect Lucifer Yellow, coronal sections were incubated with an anti-Lucifer-Yellow antibody (Sigma-Aldrich; 1:5000 dilution) for 48 h at ambient temperature in a PBS solution containing sucrose (5%), bovine serum albumin (2%), Triton (1%), and azide (0.1%). Slices were then rinsed in PBS solution and incubated for an additional 48 h with an Alexa-conjugated secondary antibody (Molecular Probes, Carlsbad, California 1:1000). Slices were mounted and coverslipped using Immunomount (Thermo scientific, Pittsburgh, Pennsylvania).

An LSM 710 meta confocal microscope (Carl-Zeiss, Göttingen, Germany) equipped with a $\times 63$ oil-immersion objective was used for dendritic spine analysis. Only second-order dendritic shafts, situated at a distance between 100 and 200 μm from the soma were considered. Spine density analysis was performed by scrolling across single z-stacks of raw images using a plug-in specifically developed for OsiriX software (http://www.osirix-viewer.com). We counted protrusions located behind each other on z-stacks whenever distinction was possible.

Acute Brain Slices

On PD90 rats were briefly anesthetized with sevoflurane and the brain rapidly harvested and placed into ice-cold oxygenated (95% O_2 and 5% CO_2) artificial cerebrospinal fluid solution (aCSF) containing 130 mM NaCl, 3.5 mM KCl, 1.25 mM NaH_2PO_4, 24 mM $NaHCO_3$, 2 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 10 mM glucose, pH 7.4 (330 mOsmol). Horizontal hippocampal slices (400 μm) were prepared, and the slices were incubated for at least 2 h in an interface chamber containing oxygenated aCSF. A single slice was then transferred to a submersion recording chamber, where it was continuously perfused (1.8–2 ml/min) with modified aCSF (2 mM $CaCl_2$, 1 mM $MgCl_2$) warmed to 31°C–32°C (Terrando *et al.*, 2013).

Electrophysiology

Field excitatory postsynaptic potential (fEPSP) was recorded with an extracellular recording pipette (filled with the modified aCSF) positioned in the stratum radiatum of CA1 area with some modifications from (Terrando *et al.*, 2013). fEPSP was evoked by stimulating Schaffer collateral-commisural axons with a bipolar concentric electrode (FHC, Bowdoin, Maine) placed in CA1-stratum radiatum. The stimulus intensity was set to approximately 60% of the intensity that triggered population spikes and was determined empirically for each slice. Before long-term potentiation (LTP) induction, we performed stimulus-response curves using a range of stimulus intensities (8–25 μA). For LTP experiments, stimuli were applied every 60 s for $\geq$ 30 min (baseline period), and LTP was induced using 3 trains of high-frequency stimulation (HFS; 100 pulses at 100 Hz applied at 20-s intervals). Data were normalized with respect to the mean values of fEPSP slope recorded during the baseline period.

5-Bromo-2-Deoxyuridine Administration

The thymidine analog 5-bromo-2-deoxyuridine (BrdU) (Roche, Mannheim, Germany, 5 mg/ml dissolved in 0.9% saline) was prepared fresh before use and injected intraperitoneally (50 mg/kg), once daily for 2 days before sevoflurane exposure for the short-term analysis and for another 2 days after sevoflurane exposure for the long-term analysis.

Immunohistochemistry

The rats were deeply anesthetized and perfusion-fixed with 5% formaldehyde in 0.1 mol/l PBS, followed by immersion-fixation in the same fixative solution overnight as described earlier (Zhu *et al.*, 2010). One hemisphere was kept in 30% sucrose in 0.1 mol/l PBS until it sank. Sagittal sections (35 μm) were cut using a vibratome and sections were stored in tissue cryoprotectant solution (25% ethylene glycol, 25% glycerol, and 0.1 mol/l phosphate buffer) at −20°C. These were used for cell proliferation (BrdU, proliferating cell nuclear antigen [PCNA]) and differentiation (BrdU/NeuN, BrdU/S100) stainings as well as 4′,6-diamidino-2-phenylindole (DAPI) staining for counting of the total number of neuronal nuclei in the granule cell layer (GCL). The other hemisphere was dehydrated, paraffin-embedded, and cut into 5-μm sections. These sections were stained for the cell death-related marker active caspase-3.

BrdU staining was performed on free-floating sections. The halogenated pyrimidine was exposed through DNA denaturation. This was achieved by incubating the sections in 2 mol/l HCl for 30 min at 37°C, rinsing in 0.1 mol/l borate buffer (pH 8.5) for 10 min, followed by several rinses in Tris-buffered saline (TBS: 0.08 mol/l Trizma-HCl, 0.016 mol/l Trizma-Base, 0.15 mol/l NaCl, pH 7.5). Sections were incubated in blocking solution for 30 min (3% donkey serum and 0.1% Triton X-100 in TBS) and then incubated with primary antibody (rat anti-BrdU, 1:500, 5 mg/ml; clone: BU1/75, Oxford Biotechnology Ltd, Oxfordshire, UK) in blocking solution for 16 h at 4°C. After rinsing with TBS, sections were incubated for 2 h at room temperature with biotinylated donkey anti-rat IgG (1:1000, Jackson ImmunoResearch Lab, West Grove, Pennsylvania), followed by avidin-peroxidase for 60 min (ABC kit, Vectastatin Elite, Vector Laboratories, Burlingame, California), and then detection solution (26.5 mg/ml diaminobenzidine, 0.01% H_2O_2, 0.04% NiCl). For the PCNA staining, sections (35 μm) were denatured in 10 mmol/l sodium citrate solution (pH 9.0) for 30 min at 80°C and endogenous peroxidase activity was quenched by 0.6% H_2O_2 for 30 min. After rinsing, the sections were blocked with 3% donkey serum and then incubated with rabbit anti-PCNA polyclonal IgG (1:500, 5 mg/ml, Upstate, Lake Placid, New York) in blocking solution at 4°C overnight, followed by 60 min at room temperature with biotinylated donkey anti-rabbit IgG antibody (1:1000, Jackson ImmunoResearch Lab). For doublecortin (DCX) staining, the endogenous peroxidase activity was removed by 0.6% H_2O_2 for 30 min. After rinsing, the sections were blocked with 3% donkey serum and then incubated with goat anti-DCX polyclonal IgG (1:300, sc-8066, Santa Cruz, Biotechnology) at 4°C overnight, followed by 60 min at room temperature with biotinylated donkey anti-goat IgG antibody (1:500, Jackson ImmunoResearch Lab).

The phenotypes of BrdU-labeled cells were determined using triple immunofluorescence staining. Antibodies against NeuN and S-100β were used to detect mature neurons and astrocytes, respectively. DNA denaturation was performed as above, followed by incubation with rat anti-BrdU (1:500, 2 mg/ml; clone: BU1/75, Oxford Biotechnology Ltd) together with mouse anti-NeuN monoclonal antibody (1:200, 5 mg/ml; clone: MAB377, Chemicon, Temecula, California) and rabbit anti-S-100β (1:500; Swant, Bellinzona, Switzerland) in TBS at 4°C. After washing, the sections were incubated with secondary antibodies, Alexa 488 donkey anti-rat IgG (H + L) combined with Alexa 555 donkey anti-mouse IgG (H + L) and Alexa 647 donkey anti-rabbit IgG (H + L) at room temperature for 2 h. All secondary antibodies

were from Jackson ImmunoResearch Lab, diluted 1:1000. Sections were mounted using Vectashield mounting medium.

Staining of the cell death-related marker active caspase-3 and the cell proliferation/survival-related marker BrdU was performed in paraffin sections as described in Zhu *et al.* (2010). Sections (5 μm) were deparaffinized and rehydrated. Antigen retrieval was performed by heating the sections in 10 mmol/l boiling sodium citrate buffer (pH 6.0) for 10 min. Nonspecific binding was blocked for 30 min with 4% goat or horse serum in PBS. Rabbit anti-active caspase-3 (1:300, 10 mg/ml, BD Pharmingen, Franklin Lakes, New Jersey), rat anti-BrdU (1:100), was incubated for 60 min at room temperature, followed by the appropriate, biotinylated goat anti-rabbit (1:200), horse anti-rat (1:200) secondary antibody for 60 min at room temperature. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 5 min. Visualization was performed using Vectastain ABC Elite with 0.5 mg/ml 3,30-diaminobenzidine enhanced with 15 mg/ml ammonium nickel sulfate, 2 mg/ml β-D glucose, 0.4 mg/ml ammonium chloride, and 0.01 mg/ml β-glucose oxidase (all from Sigma, Stockholm, Sweden).

Cell Counting

The number of BrdU-labeled cells and PCNA-positive cells was counted in the GCL, including the subgranular zone (SGZ), using unbiased stereological counting techniques (Zhu *et al.*, 2010). All cells were counted in every 12th section using a semiautomatic stereology system (Stereo Investigator, MicroBrightField Inc, Magdeburg, Germany). After outlining the borders of the GCL, the area was calculated automatically. Cells were counted in the whole area. The cells were also counted in the z plane. The counting volume was calculated by multiplying the average of all the traced areas from each section with the section thickness, times 12. The cell density was calculated from the sum of all cells counted divided by the counting volume. For the immunofluorescence staining, at least 50 BrdU-positive cells in the GCL were phenotyped using a confocal laser scanning microscope (Leica TCS SP, Heidelberg, Germany) and the ratios of BrdU/NeuN or BrdU/S100β double-labeled cells were calculated for each sample. The total number of newborn neurons (BrdU/NeuN-positive) and astrocytes (BrdU/S100β-positive) in each sample was calculated based on the number of BrdU-positive cells and the ratio of double labeling. DCX-positive cells were counted in the SGZ of the dentate gyrus (DG) and expressed as cells/mm^3. Active caspase-3-positive cells were counted in the GCL and the SGZ of the DG and the whole cornu ammonis (CA), and were expressed as total numbers of positive cells. All counting was performed by investigators blinded to group assignment.

Behavioral Tests

Open field. The open field test allows for evaluation of anxiety-related and exploration-related motor activity (Kalm *et al.*, 2013). A 1 × 1 m square arena with 30 cm high walls, made of gray polypropylene was used. The arena was placed in the center of a brightly lit room. Each rat was released in the center of the arena and allowed to explore it for 20 min. The middle of the animal's body was defined as the point for tracking zone entries. The central zone was defined as a 30 × 30 cm area in the center of the arena. Anxiety-related activity was assessed by the time spent avoiding the central zone. A recorded video was analyzed using the EthoVision 3.1 video-tracking software (Noldus Information Technologies, Netherlands) with respect to the following parameters: track length, velocity, time in motion, distance from the wall, central zone visits, and duration. The arena was cleaned with 70% alcohol after each animal.

Novel object recognition. The novel object recognition (NOR) task was tested in an open field arena by an investigator blinded to the treatment of the animals as described previously (Zhu *et al.*, 2010). The test consisted of 2 trials with a 24-h interval. During the first trial, rats were placed in the arena containing 2 identical objects for 5 min. The time spent exploring each object was video recorded. After each session, the objects were cleaned thoroughly, and the test box was cleaned using 70% ethanol. The exploration of an object was defined as rearing on the object, sniffing at it at a distance of < 2 cm and/or touching it with the nose. For the second trial, one of the objects presented in the first trial was replaced by a novel object. The rats were returned to the arena for 5 min and the total time spent exploring each object was measured. Data are expressed as recognition index, corresponding to the difference between the time spent exploring the novel and the familiar objects, divided by the total time exploring both objects.

Novel place recognition. The novel place recognition (NPR) task is a spatial recognition memory test generally considered to be hippocampus-dependent. For all phases of the NPR task, the arenas were cleaned thoroughly between animals using 70% ethanol to minimize confounding odor cues. For all trials, the investigator, who was blinded to the treatment of the animals, placed the rat in the center of the testing arena in a consistent orientation to minimize the chance that rats would simply explore the block that they happened to be placed next to. Two identical plastic blocks that had been thoroughly cleaned with 70% ethanol were placed at specific positions in the testing arenas. Rats were allowed to explore them freely for 5 min. Following the familiarization phase, the rats were placed in a different cage for 5 min. During this time, the testing arenas were cleaned with 70% ethanol, and identical copies of the blocks were added; one in an identical position as during the familiarization phase, and one in a novel spatial position. After the 5-min delay interval, the rat was returned to the testing arena for 5 min, and again after a 24-h interval. For the 24 h delay test, one block remained in the same position as during the familiarization and 5-min test phases, and the other block was placed in a novel spatial position, different from the 2 previous positions. For both the 5-min and 24-h test phases, the exploration ratio was calculated as time spent exploring the novel position minus the time spent exploring the familiar position divided by the total time spent exploring the 2 positions/objects.

Statistical Analyses

Statistical analysis was performed using the Prism 5 program (GraphPad Software). Statistical significance was assumed when $P < .05$, tested using the unpaired 2-tailed Student's *t* test or ANOVA, where applicable.

RESULTS

All animals at the 3 study centers completed the study protocol and no mortality was observed in any of the experimental groups exposed to a 30-min sevoflurane anesthesia at 2.5% inspired fraction. Moreover, we could not detect any significant changes in heart rate (HR) or oxygen saturation (SpO_2) during the experimental procedure (Figs. 1A and B; $P > .05$ all interactions, 2-way ANOVA).

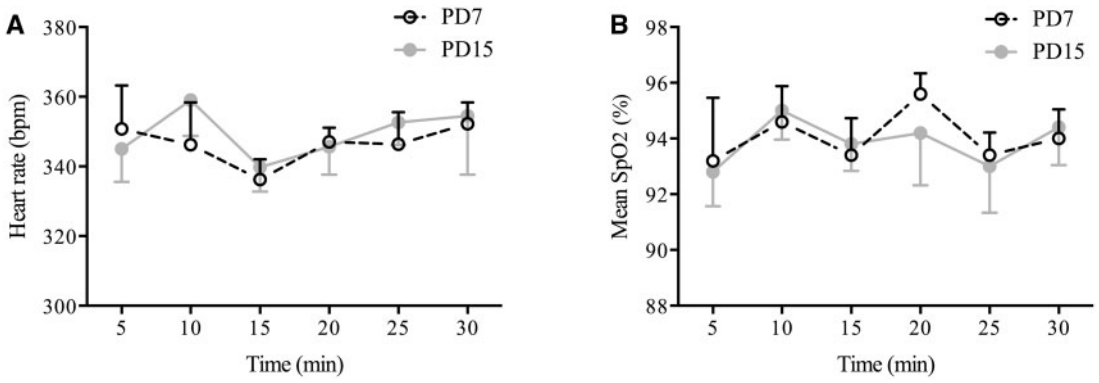

FIG. 1. Vital signs monitoring during anesthesia. Heart rate (A) and oxygen saturation (B) was similar in 7-day-old (PD7) and 15-day-old (PD15) rats during the 30 min sevoflurane anesthesia ($n = 5$/group).

Sevoflurane Induces Developmental Stage-Dependent Region-Specific Transient Alterations in Dendritic Spine Densities

To investigate the effects of sevoflurane exposure on synaptogenesis, we focused on dendritic spines of layer 2/3 pyramidal neurons in 2 distinct cortical regions as well as of CA1 principal cells in the hippocampus at PD7 and at PD15.

On PD7, exposure to sevoflurane anesthesia rapidly induced a decrease in dendritic spine densities of layer 2/3 pyramidal neurons both in the medial prefrontal ($P < .001$) and in the somatosensory ($P < .001$) cortices (Figs. 2A and B). On CA1 principal cells, where dendritic spine densities are several-fold lower compared with cortical pyramidal neurons at this developmental stage, no statistically significant differences ($P = .25$) in dendritic spines densities could be detected between control and sevoflurane-exposed animals (Figure 2C). In contrast, when the same experimental protocol was performed at PD15, sevoflurane rapidly induced a significant increase in dendritic spine densities both in the medial prefrontal ($P < .001$), somatosensory ($P = .0015$), and CA1 ($P = .047$) cerebral regions.

To investigate whether the acute developmental stage-dependent changes in dendritic spine densities persisted over time, we also quantified dendritic spines in the same cerebral regions of 3-month-old animals that received a 30-min sevoflurane exposure at PD7 or at PD15. Neither the PD7 nor the PD15 exposure groups displayed sevoflurane-induced alterations in dendritic spine densities persisting into adulthood (Figs. 2D–F), demonstrating the transient nature of changes induced by this experimental protocol.

Long-Term Effects of Sevoflurane Exposure on Hippocampal-Dependent Synaptic Plasticity in Adults

To further investigate the long-term effects of sevoflurane anesthesia on neuronal activity, we assessed basal synaptic transmission and plasticity in acute hippocampal slices at 3 months from PD7 and PD15 exposed rats.

Basal synaptic transmission was studied by analyzing stimulus-response (I/O) curves and short-term potentiation by assessing paired-pulse facilitation (PPF) and LTP at CA3-CA1 synapses. Previous exposure to 30 min sevoflurane anesthesia on PD7 or PD15 did not affect basal synaptic transmission (Figure 3A; $P = .09$). The means from the I/O curve slopes from the different groups were not significantly different (Figure 3B; $P = .09$). However, rats exposed to sevoflurane anesthesia on PD7 showed larger PPF ratio (Figure 3C; 2-way ANOVA; $P = .01$), suggesting presynaptic calcium alterations. LTP level, measured as the mean fEPSP slope at 45–55 min after HFS stimulation, was similar in all groups (Figs. 3D and E; 1-way ANOVA; $P = .56$).

Brief Sevoflurane Exposure Does Not Impair Proliferation or Differentiation in the Neurogenic Hippocampus of the Juvenile Brain

The short-term effect of sevoflurane exposure on newborn stem/progenitor cells, born (and hence BrdU-labeled) 1 or 2 days earlier, was evaluated 6 h after exposure. The number of newborn cells in the SGZ was not different between the control and sevoflurane groups, neither for the PD7 nor for the PD15 rats (not shown) (Figure 4). Extending the exposure time from 30 min to 2 or 4 h in PD7 rats, the presumed more susceptible developmental stage, did not result in reduced numbers of newborn stem/progenitor cells (Figure 4A). Together, these results demonstrate that proliferation and/or survival of newborn stem/progenitor cells were not adversely affected by 30 min sevoflurane exposure, neither at PD7 nor at PD15, and that extending the exposure time to 4 h at PD7 did not change this. The long-term effect of 30 min sevoflurane exposure on stem/progenitor cell proliferation/survival, as judged by the number of actively proliferating (PCNA-positive) cells 3 months later, was also not affected. The number of actively proliferating, PCNA-positive cells in the SGZ was not different between control and sevoflurane exposure, neither in the PD7 nor in the PD15 groups (Figure 4B). Neurogenesis, as judged by the number of DCX-positive cells 3 months after exposure, was also not different between controls and sevoflurane exposed rats, neither in the PD7 nor in the PD15 groups (Figure 4C). These results clearly demonstrate that brief sevoflurane exposure did not affect neural stem/progenitor proliferation, survival or differentiation, neither shortly after (6 h) nor in adulthood (3 months later).

To investigate the effects of sevoflurane exposure on the survival and differentiation of newborn cells (born from 2 days prior to exposure up to 2 days after exposure), rats received BrdU injections once daily for 2 successive days before sevoflurane exposure and 2 successive days after sevoflurane exposure. The number BrdU-labeled cells in the GCL (including the SGZ) was higher in the PD7 group than that of PD15 group, as expected (Figs. 5A and B), but no difference was observed after sevoflurane exposure, neither in the PD7 nor in the PD15 groups (Figure 5D). Neurogenesis and astrogenesis, as judged by the numbers of BrdU/NeuN- and BrdU/S100-positive cells, respectively, in the DG 3 months after sevoflurane exposure were not different, neither in the PD7 nor in the PD15 groups (Figs. 5C, E, and F).

Brief Sevoflurane Exposure Does Not Increase Cell Death in the Hippocampus

Cell death was detected by staining for active caspase-3 6 h after sevoflurane exposure in both PD7 and PD15 rats. Active

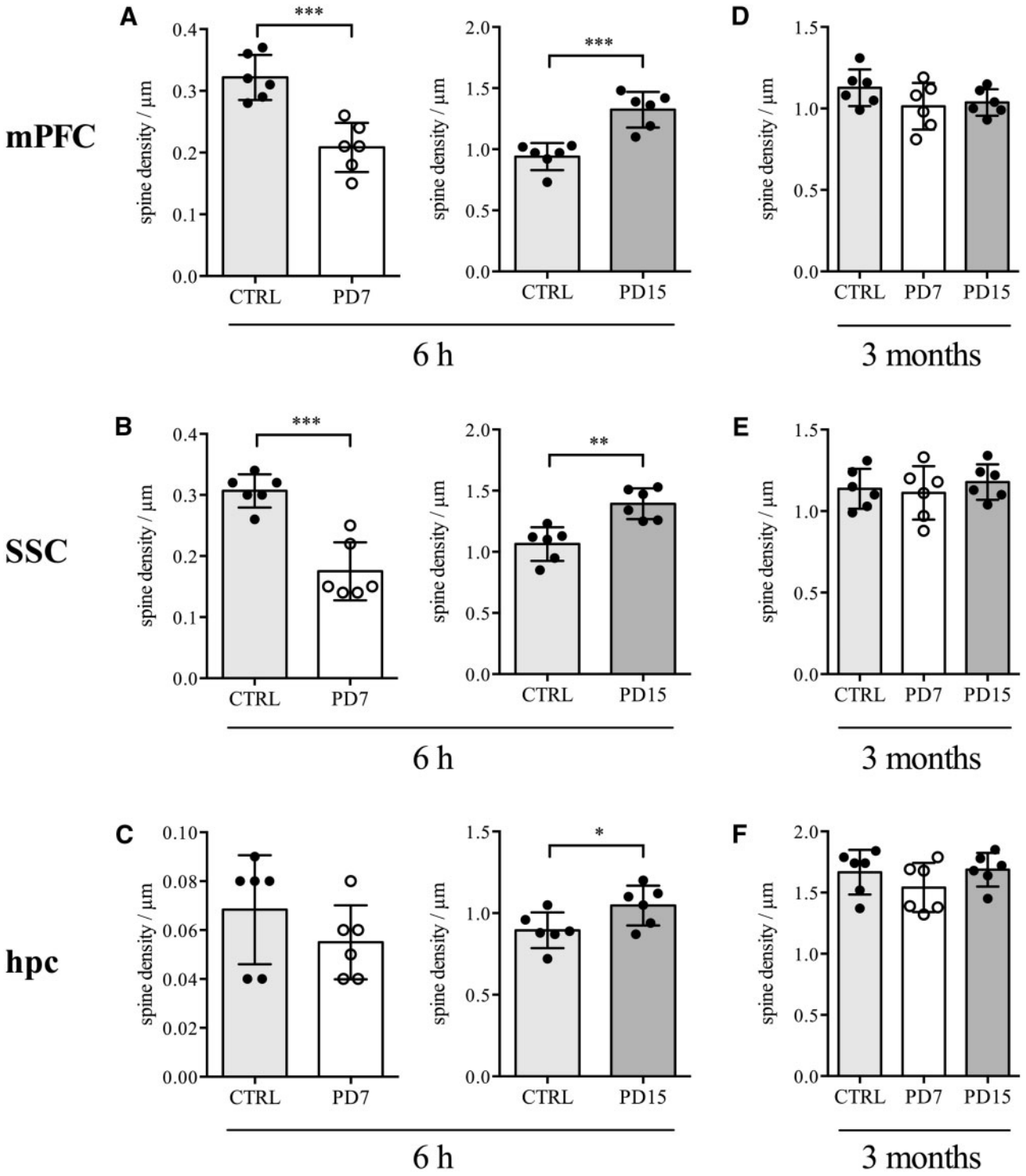

FIG. 2. Developmental stage-dependent and region-specific effects of sevoflurane on dendritic spinogenesis. Quantitative analysis of dendritic spine densities on apical dendrites of layer 2/3 pyramidal neurons in the mPFC (A), SSC (B) or on CA1 (C) pyramidal cells 6 h following the 30-min-long sevoflurane exposure shows decreased spine densities in PD7 animals but increased spine densities when sevoflurane is applied at PD15. D–F, These acute changes do not persist into adulthood in any of these brain regions ($n = 6$/group). Abbreviations: mPFC, medial prefrontal cortex; SSC, somatosensory cortex; hpc, hippocampus. ***$P < .001$; **$P = .0015$; *$P = .047$

caspase-3-positive cells could be detected scattered throughout the hippocampus under normal conditions, and the numbers were higher in the CA region than in the GCL and SGZ of the DG of PD7 rat pups, as expected from the higher turnover of cells at this developmental stage under control conditions. The numbers of active caspase-3-positive cells were not different between the groups after 30 min sevoflurane exposure, neither in the PD7 nor in the PD15 group (Figs. 6A and B). After 4 h exposure, however, an increased number of active caspase-3-positive cells were observed in the CA region ($P < .05$) but not in the GCL or the SGZ in the PD7 rats.

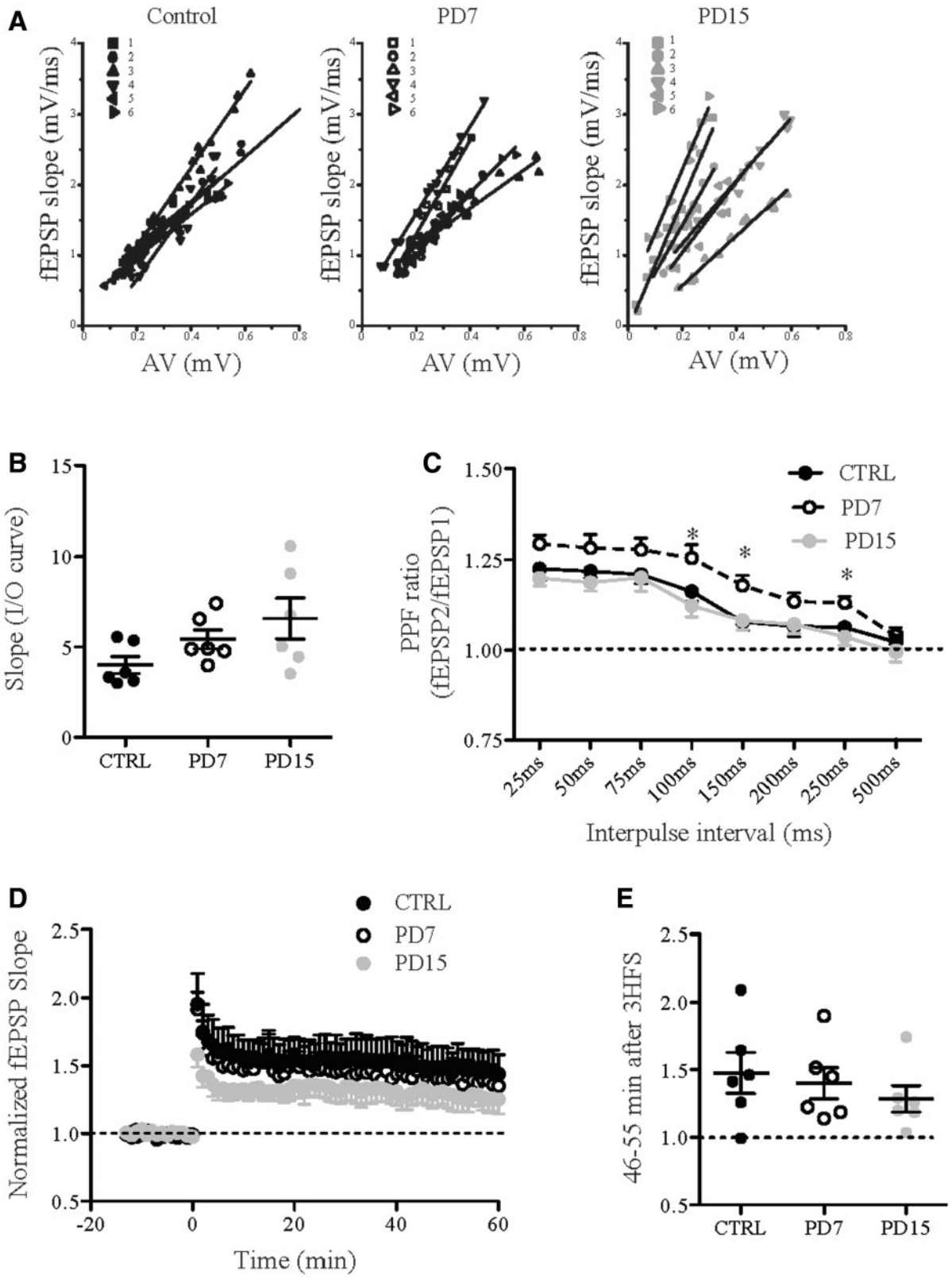

FIG. 3. Effects on long-term synaptic transmission and plasticity. A, I/O curves representing individual experiments (1–6) from each group (control [CTRL], PD7, and PD15). B, Average I/O curves were created by adjusting the individual responses (panel A) to a linear curve and the slopes from each individual experiment plot in form of averaged slope. C, Mean PPF ratio at the different interpulse intervals applied (25–500 ms) during the experiment. Data are represented as the ratio of the second fEPSP slope relative to the first fEPSP slope. D, LTP of CA3-CA1 pyramidal cells synapses. Three trains of HFS (100 Hz; 20-s interval) were applied at 0. The evoked fEPSP were normalized against the baseline period (−30 to 0 min). E, Mean of the fEPSP slope recorded at 45–55 min after the application 3HFS ($n = 6$ slices from 3 animals/group). Abbreviations: I/O, input/output curve; fEPSP, field excitatory postsynaptic potential; AV, average volts; PPF, paired-pulse facilitation; LTP, long-term potentiation; CA, cornu ammonis; HFS, high-frequency stimulation. *$P < .05$

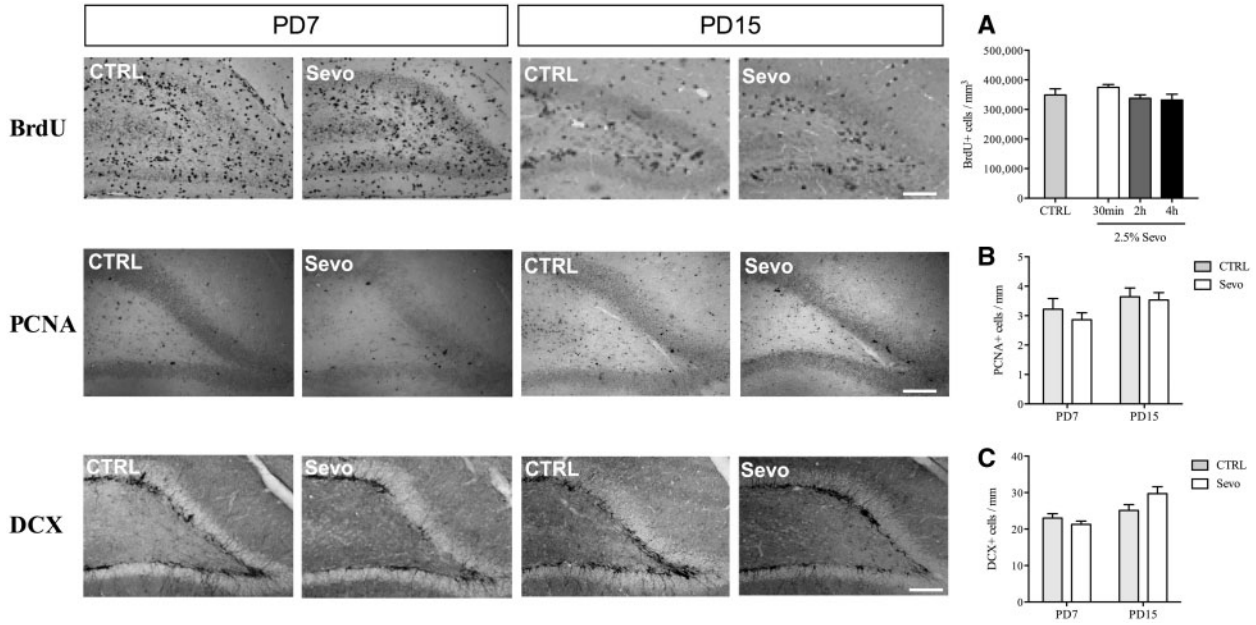

FIG. 4. Effects of sevoflurane on proliferation and neural differentiation in the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus. A, Representative images of 5-bromo-2-deoxyuridine (BrdU) labeling in CTRL and sevoflurane-exposed (Sevo) on PD7 and PD15, respectively. The BrdU-labeled cells were quantified in the SGZ 6 h after exposure and the numbers were not different between the groups, neither in PD7 rats ($n = 9$ for control and $n = 10$ for sevoflurane) nor in PD15 rats ($n = 9$ per group). B, Representative images of proliferating cell nuclear antigen (PCNA) labeling in the CTRL and Sevo groups from PD7 and PD15 rats. There were no differences in the numbers of PCNA-positive cells in the SGZ 3 months after exposure, neither in PD7 nor in PD15 rats ($n = 10$ for CTRL and $n = 15$ for Sevo). C, Representative images of doublecortin (DCX) labeling (reflecting neurogenesis) in the CTRL and Sevo groups from PD7 and PD15 rats. There were no differences in the numbers of DCX-positive cells in the SGZ 3 months exposure, neither in PD7 nor in PD15 rats ($n = 10$ for CTRL and $n = 15$ for Sevo). Scale bar = 150 μm.

No Impairments in NOR and NPR Paradigms in Animals Exposed to Brief Sevoflurane Anesthesia

To corroborate the results from the biochemical assays and electrophysiology, we assessed memory function in PD7 and PD15 rats 3 months after sevoflurane exposure using NOR and NPR paradigms (Figure 7A). We also assessed the overall motor activity using an open field test. In the NOR test, the recognition index was not different between control and sevoflurane-exposed rats 3 months after exposure, neither for the PD7 groups nor for the PD15 groups (Figure 7B). In the NPR test, the short-term (5 min) memory (Figure 7C) and long-term (24 h) memory (not shown) were assessed. The recognition index was not different between controls and sevoflurane-exposed rats at any of the time points or any of the intervals between training and testing, neither in PD7 nor in PD15 rats. In the open field test, no apparent differences were found in time spent in motion (Figure 7D), track length, distance from the walls, or the number of visits to or time spent in the central zone between control and sevoflurane exposed rats, neither for the PD7 nor for the PD15 groups (not shown).

DISCUSSION

The present work aimed to bridge the current gap of knowledge as to whether a short exposure to the general anesthetic sevoflurane during early postnatal life impacts later brain development. By focusing on 2 functionally distinct stages of the rat brain growth spurt and by combining a series of biochemical, histological, electrophysiological, and behavioral approaches, we investigated both the short- and long-term impact of a 30-min sevoflurane exposure at clinically relevant concentrations on developing brain circuitry. We show that while physiological parameters remained stable during anesthesia, sevoflurane rapidly induced developmental stage-dependent, region-specific, transient changes in dendritic spine densities. Electrophysiological investigations revealed a significant increase in PPF in the hippocampus of adult rats that were exposed to sevoflurane at PD7 but not at PD15. In contrast, no changes in hippocampal LTP were found at any experimental group when evaluated 3 months following drug exposure. BrdU pulse and pulse-chase experiments revealed that neither the proliferation nor the differentiation and survival of hippocampal progenitors were impaired upon sevoflurane exposure at either developmental stage. Finally, a series of cognitive tests destined to assess short- and long-term memory revealed no differences between control and sevoflurane-exposed animals. Additionally, the overall motor behavior in a novel environment was not different after sevoflurane exposure at either age, indicating that anxiety-related behavior was not affected. Altogether, these data suggest that brief sevoflurane exposure in the early postnatal period does not seem to exert major morpho-functional changes on CNS development. However, our observations also raise the possibility that more subtle changes in synaptic plasticity might persist following drug exposure, of which the physiological significance remains to be determined.

We focused on sevoflurane because this agent is amongst the most commonly used anesthetics in neonatal and pediatric anesthesia practice (Lerman *et al.*, 1994). Hence, detailed evaluation of how this drug, at clinically relevant doses and exposure lengths, affects CNS development is of utmost importance. To achieve this aim, sevoflurane was administered at a concentration of 5% until the loss of the righting reflex, and then was maintained at 2.5% throughout the whole duration of anesthesia exposure. These values are somewhat inferior to minimum alveolar concentration (MAC) of sevoflurane in neonatal rodents (Orliaguet *et al.*, 2001) but induce complete loss of the righting reflex at the 2 experimental time point investigated. Moreover, dosing regimens used herein, are very similar to what is

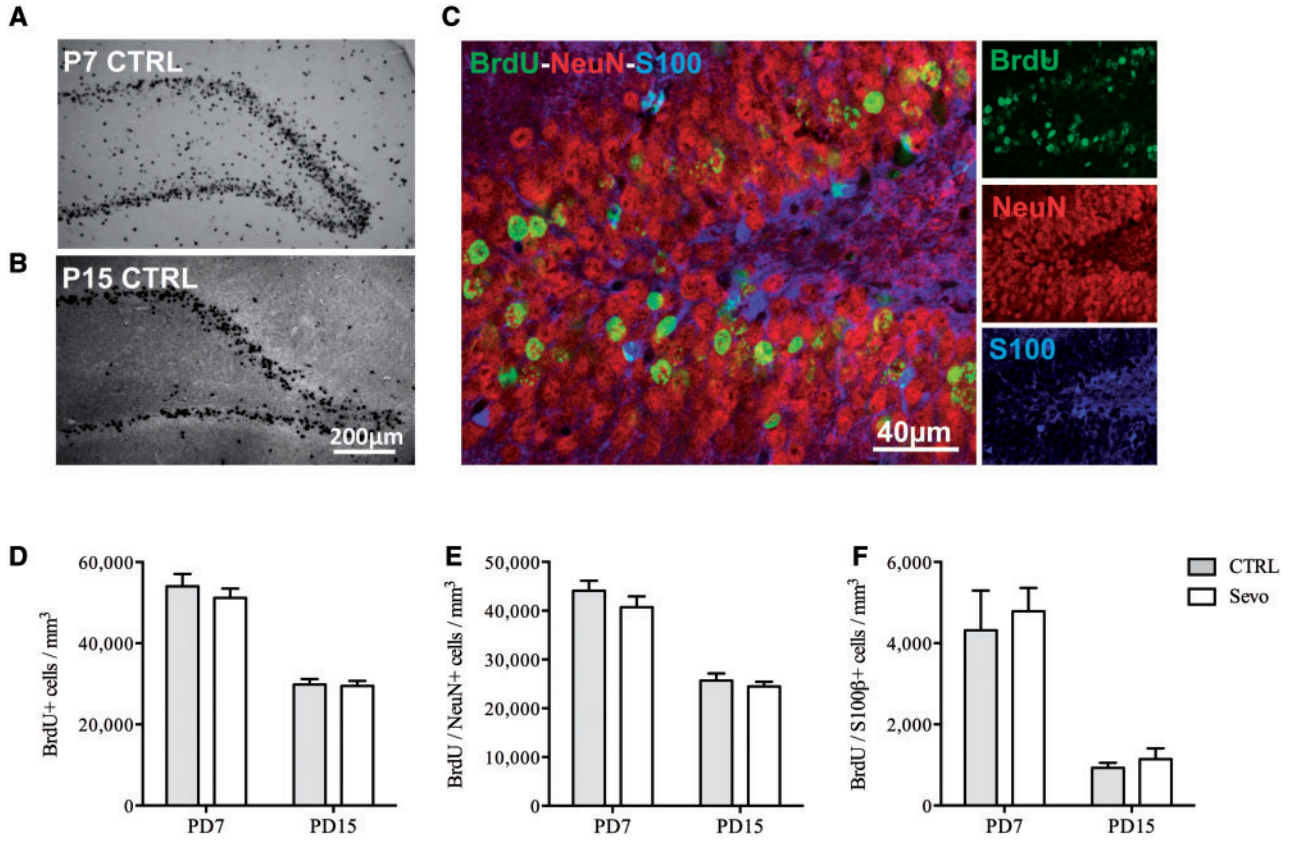

FIG. 5. Effects of sevoflurane on cell survival and differentiation in the DG. A and B, Representative BrdU stainings from the DG of CTRL PD7 and PD15 rats 3 months after exposure. C, Representative BrdU (green)-NeuN (red)-S100 (blue) staining. D, The BrdU-labeled cells were quantified in the granule cell layer (GCL) of the DG 3 months after 30 min exposure and the numbers were not different between the groups, neither in PD7 nor in PD15 rats. E, The BrdU/NeuN-labeled cells (newborn neurons) were quantified in the GCL 3 months after 30 min exposure and the numbers were not different between the groups, neither in PD7 nor in PD15 rats. F, The BrdU/S100β-labeled cells (newborn astrocytes) were quantified in the GCL 3 months after 30 min exposure and the numbers were not different between the groups, neither in PD7 nor in PD15 rats. ($n = 10$ for CTRL and $n = 15$ for Sevo).

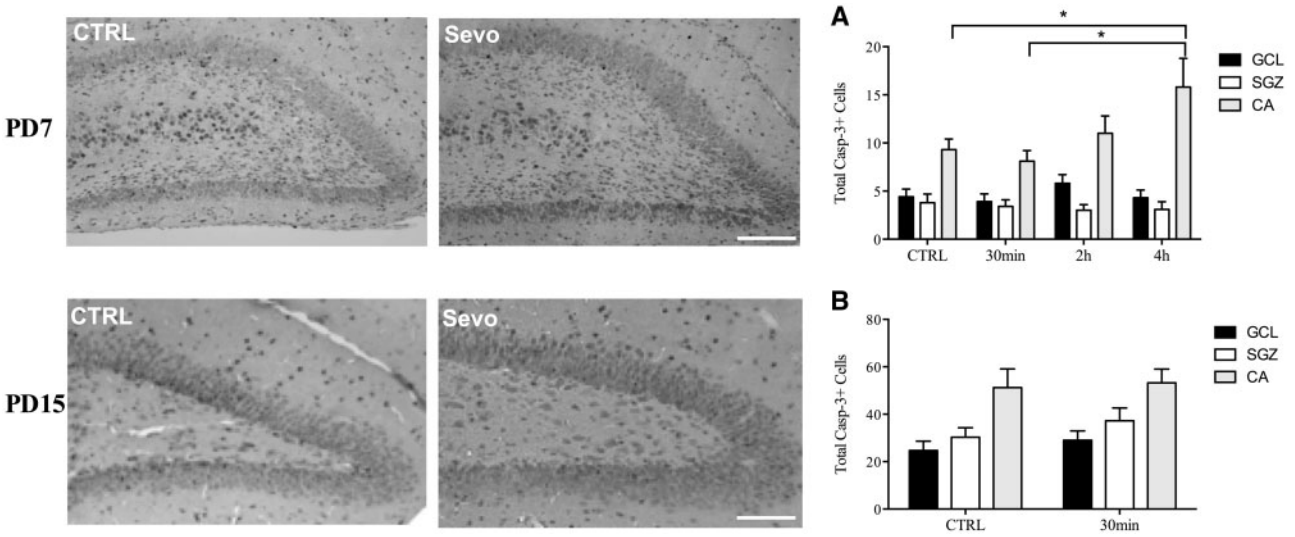

FIG. 6. Cell death in the DG 6 h after sevoflurane exposure. A, Representative images of active caspase-3 staining in the DG 6 h after exposure in CTRL and sevoflurane-exposed (Sevo) PD7 rats. The bar graph shows the quantification of active caspase-3 positive cells in the GCL and the SGZ of the DG, as well as the CA 6 h after sevoflurane exposure in PD7 rats. The numbers were not different between the groups after 30 min or 2 h exposure, but after 4 h exposure the numbers were higher in the CA ($n = 9$ for CTRL and $n = 10$ for Sevo). B, Representative images of active caspase-3 staining in the DG 6 h after exposure in CTRL and sevoflurane-exposed (Sevo) PD15 rats. The numbers were not different between the groups after 30 min exposure in any of the 3 regions ($n = 9$/group). Scale bar = 150 μm. *$P < .05$.

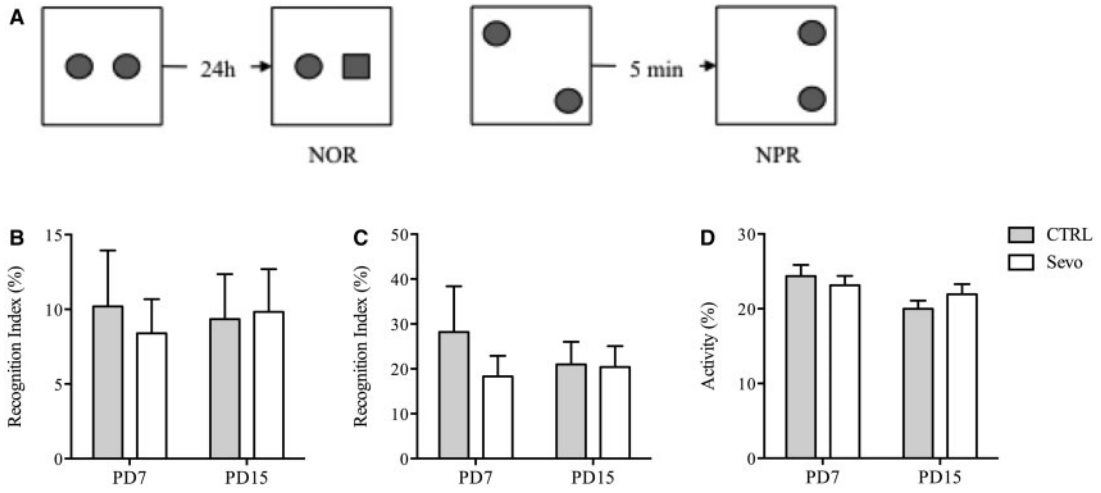

FIG. 7. Behavioral tests. A, The experimental designs of novel object recognition (NOR) and novel place recognition (NPR). B, NOR was performed 3 months after sevoflurane exposure. No differences in the recognition index were observed, neither in PD7 nor in PD15 rats. C, NPR was performed 3 months after sevoflurane exposure. No significant differences in the recognition index were observed, neither in PD7 nor in PD15 rats. D, An open field test was performed 3 months after sevoflurane exposure. The activity (time spent in motion out of the 20 min) was not different, neither in PD7 ($n = 10$ for CTRL and $n = 15$ for Sevo) nor in PD15 ($n = 15$/group) rats.

commonly used in children (Lerman *et al.*, 1994), and, therefore, might presents valuable translational relevance. In an attempt to get closer to the clinical situation, HR and peripheral SpO_2 were closely monitored in pups throughout the anesthesia procedure and we maintained in the normal physiological range. This issue is important since recent studies raise the possibility that anesthetics-induced changes in the developing brain, especially in young animals with low body weight, may reflect considerable alterations of systemic homeostasis by these drugs rather than drug-induced direct neurotoxicity *per se* (Deans *et al.*, 2015; Wu *et al.*, 2014). Thorough monitoring of systemic parameters in these experiments is therefore of utmost importance to exclude such confounders (Vutskits and Patel, 2014).

To answer the question of whether and how short exposure to sevoflurane affects developing neuronal circuitry, we chose a 30-min-long exposure and used a wide range of experimental approaches to investigate the neurotoxic potential of this experimental paradigm. The rationale behind this particularly short exposure time was 2-fold. The first is related to the extrapolation of drug exposure durations between rodents and humans. Indeed, while the brain growth spurt occurs over a period of several months or even years in humans, it is limited to a period of approximately 3 weeks in rodents. Thus, one might argue that a several-hours-long anesthesia exposure in rodent pups might correspond to weeks of drug exposure in humans (Soriano *et al.*, 2005). Also, amongst those infants and children who needs anesthesia for surgery or other diagnostic procedures during early life, a substantial proportion is exposed to anesthetics only for a limited period of time, rarely exceeding 2 h. Therefore, we believe that evaluating the impact of a 30-min-long sevoflurane exposure in neonatal and juvenile pups is a relevant issue. The second reason to choose this short exposure time period was that we have recently demonstrated that this experimental paradigm, when performed in PD15 pups, could rapidly induce a significant increase in dendritic spine densities of layer 5 pyramidal neurons in the medial prefrontal cortex (Briner *et al.*, 2010). The idea behind evaluating the effects of sevoflurane at 2 distinct developmental time points in the early postnatal period was dictated by the important changes in growth factor signaling, neurotransmitter receptor compositions and transmission modalities between early and later stages of the brain growth spurt (Fritschy *et al.*, 1994; Kaila *et al.*, 2014; Lujan *et al.*, 2005; Semple *et al.*, 2013). We focused on PD7 and on PD15 because these time points, respectively, represent early and later stages of the peak synaptogenic period in rodents (Briner *et al.*, 2011; De Felipe *et al.*, 1997). It is very difficult to precisely extrapolate the relevance of these developmental time points in rats to human brain development given the cell type- and brain region-specific differences in maturation patterns between these 2 species (Semple *et al.*, 2013). Nevertheless, based on comparisons of neuronal architecture, it has been proposed that the PD7 in rats might represent the last trimester of human pregnancy while the PD15 time point in these animals might correspond to the first few postnatal years in humans (Clancy *et al.*, 2007; Semple *et al.*, 2013).

We demonstrate that a 30-min-long exposure to sevoflurane rapidly induced developmental stage-dependent and region-specific alterations in dendritic spine densities. These data are in line with our previous observations demonstrating developmental stage-dependent effects of propofol on dendritic spines of pyramidal neurons in the medial prefrontal cortex (Briner *et al.*, 2011). Because both propofol and sevoflurane are positive allosteric modulators of the gamma-Aminobutyric acid ($GABA_A$) receptor (Rudolph and Antkowiak, 2004), and since signaling through this receptor complex undergoes a gradual transition from excitatory toward inhibitory modalities during the brain growth spurt (Kaila *et al.*, 2014), a plausible explanation for these observed developmental stage-dependent effects could be provided by the functional switch of GABAergic neurotransmission, primarily linked to the developmental upregulation of the cation-chloride cotransporter KCC2 (Kaila *et al.*, 2014). In line with this hypothesis, we and others have shown that KCC2 is up-regulated in the developing rat brain between PD7 and PD15 and that this cation cotransporter is involved in dendritic spinogenesis and synapse formation (Fiumelli *et al.*, 2013; Kaila *et al.*, 2014; Lacoh *et al.*, 2013; Li *et al.*, 2007). Most importantly, recent works from our laboratory demonstrated that premature functional switch toward the inhibitory functional modalities of GABAergic neurotransmission, protects against anesthetics-induced dendritic spine loss durng the early postnatal period (Fiumelli *et al.*, submitted). Although sevoflurane rapidly triggered changes in dendritic spine densities both in the medial

prefrontal and in the somatosensory cortex, no such changes have been found on pyramidal neurons in the CA1 region of the hippocampus. The mechanisms underlying these region-specific differences remain to be determined but might reflect differences in connectivity patterns as well growth factor and/or receptor signaling pathways.

An important observation of this study was that the brief sevoflurane exposure-induced acute changes in dendritic spine densities did not persist over time. These data are in contrast with recent observations from our own as well as from other laboratories indicating persistent changes in neuronal circuitry following 6-h-long exposure to anesthetics (Briner *et al.*, 2011; Lunardi *et al.*, 2010), and suggest that the duration of exposure might play an important role regarding long-term toxicity. The mechanisms explaining why anesthetics-induced acute changes in dendritic spines are lasting after prolonged but not after brief exposure are as yet unexplained. Also, we cannot exclude that the brief sevoflurane exposure-induced acute transient changes in dendritic spine densities will give rise to transient or even lasting changes in functional network properties. In favor of this possibility, our data revealed larger PPF in acute hippocampal slices of 3-month-old rats after exposure to sevoflurane at PD7. These findings suggest that early anesthesia exposure induced some lasting effects of neural transmission, at least at the presynaptic level. It is possible that the changes we observed in PPF are due to an increase in presynaptic calcium concentrations, which has been proposed as a possible mechanism in anesthesia-induced neurotoxicity (Joseph *et al.*, 2014). However, in this model we could not find significant changes in hippocampal-evoked LTP following a 30-min-long sevoflurane exposure. These observations, together with recent data showing impaired LTP after a 2-h-long exposure to sevoflurane (Kato *et al.*, 2013), strongly suggest an important role for the length of exposure to induce significant lasting changes in circuitry function. In line with this issue, recent observations show that a single 6-h-long or a protocol of 3 repeated 2-h-long exposures to sevoflurane in PD7 rats to sevoflurane had lasting effects on synaptic density while no such effects were observed following a single 2-h-long exposure (Amrock *et al.*, 2015).

Our treatment paradigm on hippocampal cell death, proliferation, or neurogenesis did not reveal any effect of brief sevoflurane exposure on these parameters. In light of existing data, showing that a several-hour-long exposure to anesthetics can impair neurogenesis and cell survival (Hofacer *et al.*, 2013; Stratmann *et al.*, 2009), these observations further argue for the role of exposure length to detect such effects. Indeed, as demonstrated in our present work, we also found increased apoptotic rates in the hippocampus following a 4-h-long exposure. Previous work showed an association between anesthesia-induced learning deficits and impaired neurogenesis (Zhu *et al.*, 2010). In that study, repeated exposures to 35 min isoflurane for 4 successive days revealed that object recognition as well as reversal learning were impaired in young rats and mice, whereas adult animals were unaffected, and that these deficits became more pronounced as the animals grew older. The memory deficit was paralleled by a decrease in the hippocampal stem cell pool and persistently reduced neurogenesis, subsequently causing a reduction in the number of DG granule cell neurons in isoflurane-treated rats (Zhu *et al.*, 2010). In line with the lack of effect of brief sevoflurane exposure on hippocampal neurogenesis, our behavioral paradigms could not detect any changes between control and sevoflurane-exposed animals. It is nevertheless important to note that recent observations strongly suggest a temporal relationship between exposure length and cognitive impairment, including changes in emotional behavior in monkeys (Raper *et al.*, 2015; Shen *et al.*, 2013a,b). Finally, it is worth to emphasize that behavioral paradigms applied in these as well as in our own study might only depict important changes in cognition and/or behavior while more subtle deficits might remain unnoticed.

In conclusion, this study provides evidence that brief exposures of sevoflurane during the brain growth spurt can rapidly induce developmental stage-dependent and brain region-specific changes in dendritic spine densities. Although these acute changes do not persist, electrophysiological recordings show impaired-PPF but not LTP in adult animals receiving anesthesia during the early postnatal period. The lack of brief sevoflurane exposure on hippocampal neurogenesis, cell death and behavior suggest that this short treatment paradigm might not give rise to persisting major impairment of the CNS. Further work is, however, needed to elucidate the physiological relevance of the persisting subtle impairments in synaptic plasticity described herein.

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