Role of Steroids in Hyperexcitatory Adverse and Anesthetic Effects of Sevoflurane in Neonatal Rats

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Key Words

Steroids · Seizures · Sevoflurane · Neonatal anesthesia · Estradiol

Abstract

Recent studies have demonstrated that long-term developmental effects of neonatal anesthesia were more prominent in males. We tested whether steroids, in general, and sex steroids, in particular, are involved in the mediation of sevoflurane-caused paradoxical cortical seizures during the early postnatal period. **Methods:** Cortical electroencephalograms, hippocampal synaptic activity, serum levels of steroids and the loss of the righting reflex (LORR), a marker of anesthetic effect, were measured on postnatal days 4–6 in Sprague Dawley rats of both genders exposed to 2.1% sevoflurane. **Results:** Episodes of seizures, persistent spikes in electroencephalograms and increases in serum corticosterone were similar in both genders. In the order of increasing potency, the corticosteroid receptor antagonist RU 28318, the estradiol receptor antagonist ICI 182780 and the estradiol synthesis inhibitor formestane decreased sevoflurane-induced seizures. Exogenous estradiol increased sevoflurane-caused seizures, spikes and serum levels of corticosterone. These estradiol-enhanced seizures and spikes were depressed by

ICI 182780 and the NKCC1 inhibitor, bumetanide, while RU 28318 decreased seizures only. In hippocampal CA1 neurons, estradiol increased the amplitude, rise time and area under the curve of gamma-aminobutyric acid type A receptor (GABA_AR)-mediated miniature postsynaptic currents. Exogenous estradiol shortened, while ICI 182780 and formestane lengthened the time needed for sevoflurane to induce LORR. **Conclusion:** These findings provide evidence for gender-independent acute electroencephalographic effects of sevoflurane at this age. Corticosterone and estradiol are involved in the mediation of sevoflurane-induced seizures. Estradiol, but not corticosterone, also contributes to sevoflurane-caused spikes, by enhancing GABA_AR-mediated excitation in the cortex. By increasing GABA_AR-mediated inhibition in more mature caudal regions of the brain, estradiol contributes to sevoflurane-induced LORR.

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Introduction

Our recent experimental findings demonstrated that exposure of neonatal rats to propofol caused multifold increases in the secretion of the corticosteroid hormones corticosterone and aldosterone at the time of anesthesia

Fig. 1. Illustration of the experimental protocols involving the investigation of the electroencephalographic effects of sevoflurane.

in both genders, while long-term impairment in sensorimotor gating function (reduction in the prepulse inhibition of the startle response) was observed in male, but not female, adult rats exposed to propofol as neonatal rats [1, 2]. Greater susceptibility of male rats to developmental effects of neonatal anesthesia with isoflurane and phenobarbital were reported by other laboratories [3, 4]. Interestingly, Lee et al. [4] found that isoflurane, when administered to postnatal day (P) 7 rats, acutely caused neuronal death in both genders, but long-term developmental effects of isoflurane, such as impaired object recognition and impaired social memory, were evident in male rats only. The existence of a gender dependence suggests ways to focus on the search for a mechanistic explanation of the developmental effects of neonatal anesthesia.

Steroids, in general, and sex steroids, in particular, such as estrogen and estradiol, seem to be plausible mediators of such developmental effects. Estradiol is at its highest levels during the perinatal period; its levels in the cortex are similar in male and female rats, but its levels in the hypothalamus are higher in male than female rats at this age [5, 6]. Estradiol positively modulates corticosterone levels and gamma-aminobutyric acid (GABA)ergic transmission [7–11]. Available data suggest that GABA type A receptor (GABA_AR)-mediated excitation and elevated systemic corticosterone levels at the time of anesthesia may contribute to the mechanisms that not only initiate the long-term adverse developmental effects of propofol, but also underlie propofol-caused acute electroencephalographic seizures [1, 2]. Consequently, probing the mechanisms of anesthetic-caused electroencephalographic cortical seizures may provide a key to understanding the mechanisms underlying long-term developmental abnormalities induced by anesthesia in neonates.

With this in mind, we investigated the paradoxical acute hyperexcitatory electroencephalographic effects of

sevoflurane in P4, 5 or 6 male and female rats and the roles of steroids, with a particular focus on estradiol, in mediating these effects.

Materials and Methods

Animals

P4–6 Sprague Dawley rats of both genders were studied. All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee. Pups from several litters were used for each treatment condition. At the beginning of each experiment, the pups were determined to be well nourished as judged by their stomachs being full of milk (detectable through the transparent abdominal wall).

Study Design and Anesthesia Regimen

All studies using electroencephalography (EEG) involved a single 60-min general anesthetic exposure to sevoflurane (fig. 1). Electroencephalographic recordings started after recovering from anesthesia for electrode implantation and lasted for 1 h before initiation of anesthesia with sevoflurane (baseline) and continued for another hour during anesthesia with sevoflurane – 6% sevoflurane for 3 min for anesthesia induction and 2.1% sevoflurane for 57 min for anesthesia maintenance. Electroencephalogram recordings were performed in a thermostated chamber (+37°C) with a continuous supply of oxygen (1.5 liters/min). Anesthesia gas monitoring was performed using a calibrated Datex side stream analyzer (Datex-Ohmeda, Helsinki, Finland), which sampled from the interior of the animal chamber. According to Orliaguet et al. [12], 2.1% sevoflurane lies near the 0.6 minimum alveolar concentration for P4–P6 rats. Using the chamber-equilibrated dose of 2.1% sevoflurane, the pups did not exhibit a righting reflex. Previously, we had shown that blood glucose and gas levels after 1 h of anesthesia with 2.1% sevoflurane were in the normal range [13]. All animals were without cyanosis and breathing regularly during anesthesia exposure. A rectal temperature probe was placed in some animals to monitor body temperature.

Treatments or vehicles were administered 30 min prior to anesthesia with sevoflurane to P4–P6 rat pups. The following treatment groups were investigated: (1) vehicle prior to anesthesia with sevoflurane to male pups (n = 10); (2) vehicle prior to anesthesia

with sevoflurane to female pups ($n = 11$); (3) the estrogen receptor agonist β -estradiol (estradiol; 0.1 mg/kg, s.c.) prior to anesthesia with sevoflurane to male pups ($n = 8$); (4) estradiol (0.1 mg/kg, s.c.) prior to anesthesia with sevoflurane to female pups ($n = 7$); (5) the mineralocorticoid receptor antagonist RU 28318 (10 mg/kg, i.p., $n = 9$); (6) the estradiol receptor antagonist ICI 182780 (0.05 mg/kg, s.c., $n = 11$); (7) the estradiol synthesis inhibitor formestane (2 mg/kg, s.c., $n = 10$); (8) estradiol (0.1 mg/kg, s.c.) plus the NKCC1 inhibitor bumetanide (1.82 mg/kg, i.p., $n = 8$); (9) estradiol (0.1 mg/kg, s.c.) plus ICI 182780 (0.05 mg/kg, s.c., $n = 6$); (10) estradiol (0.1 mg/kg, s.c.) plus RU 28318 (10 mg/kg, i.p., $n = 8$).

All study agents were dissolved in DMSO, except bumetanide, which was dissolved in saline. Therefore, equal volumes of DMSO or saline were used as vehicle. DMSO at these volumes does not cause any detectable effects on hyperexcitatory EEG effects of anesthetics in neonatal rats [2].

To assess serum levels of corticosterone and estradiol in conditions identical to those used to study the electroencephalographic effects of sevoflurane, separate groups of P4–P6 rat pups underwent surgery for electrode implantation and 1-hour separation from their dams before initiation of anesthesia with sevoflurane for 1 h. Treatments or vehicles were administered 30 min prior to anesthesia with sevoflurane. Blood samples were collected at 1 h of anesthesia with sevoflurane. The following treatment groups were investigated: (1) vehicle, no anesthesia ($n = 6$); (2) vehicle prior to anesthesia with sevoflurane ($n = 4$); (3) ICI 182780 (0.05 mg/kg, s.c.) prior to anesthesia with sevoflurane ($n = 4$); (4) estradiol (0.1 mg/kg, s.c.) prior to anesthesia with sevoflurane ($n = 5$).

To test whether estradiol may enhance the hyperexcitatory electroencephalographic effects of sevoflurane by enhancing GABA_AR activity, the effects of estradiol on GABA_AR-mediated miniature postsynaptic currents (mPSCs) in P4–P6 hippocampal CA1 pyramidal neurons were studied ($n = 7$). To test whether the estradiol receptors mediate estradiol-caused enhancement of GABA_AR activity, the effects of the estradiol receptor antagonist ICI 182780 on estradiol-enhanced GABA_AR-mediated mPSCs were evaluated ($n = 3$).

To obtain additional indirect evidence that estradiol may enhance the hyperexcitatory electroencephalographic effects of sevoflurane by enhancing GABA_AR activity, the role of estradiol in sevoflurane-caused loss of the righting reflex (LORR) was studied in P4–P6 rat pups. The righting reflex is predominantly mediated by midbrain neuronal networks as evident from its preservation in decerebrate rodents [14, 15]. If estradiol enhances the hyperexcitatory cortical electroencephalographic effects of sevoflurane by enhancing GABA_AR-mediated excitation, then estradiol should promote sevoflurane-caused LORR by enhancing GABA_AR-mediated inhibition in the midbrain structures of P4–P6 rat pups. In order to measure the latency to LORR, a rat pup was placed in a thermostated chamber filled with 3.5% sevoflurane. Sevoflurane supply to the chamber and measurement of sevoflurane levels in the chamber were performed as described above. The vehicle or treatments were administered 30 min prior to the placement of the rat into the chamber filled with sevoflurane. 3.5% sevoflurane lies near the 1 minimum alveolar concentration for P4–P6 rats [12]. We chose to use 3.5% sevoflurane for the LORR studies, because with sevoflurane concentrations of 6 and 2.1% that we used for the induction and maintenance of anesthesia in the electroencephalographic studies, LORR was too fast and too slow, respectively.

The following treatment groups were investigated: (1) vehicle prior to anesthesia with sevoflurane ($n = 5$); (2) estradiol (0.1 mg/

kg, s.c.) prior to anesthesia with sevoflurane ($n = 5$); (3) ICI 182780 (0.05 mg/kg, s.c.) prior to anesthesia with sevoflurane ($n = 6$); (4) formestane (2 mg/kg, s.c.) prior to anesthesia with sevoflurane ($n = 5$).

Electroencephalogram Recordings

Electroencephalogram recordings were performed in a thermostated chamber (+37°C) with a continuous supply of oxygen (1.5 liters/min).

To study cortical electrical activity in P4–P6 rat pups, animals were instrumented for electroencephalogram recording as described previously [2, 13, 16]. In brief, during a 12- to 15-min minor surgical procedure performed under isoflurane anesthesia (1.6–2.0%), 4 electrodes were implanted in bilateral occipital and frontal regions of the rat pup skull with the left frontal electrode serving as the reference electrode. Electroencephalogram recordings were performed using an electroencephalogram/electromyogram system (Pinnacle Technology, Lawrence, Kans., USA). Acquisition of the electroencephalogram was performed using the Sirenia software (Pinnacle Technology). The sampling interval per signal was 200 μ s (5 kHz). Sirenia Score (Pinnacle Technology), Clampfit 9.2 (Axon Instruments, Union City, Calif., USA) and MiniAnalysis (Synaptosoft Inc., Fort Lee, N.J., USA) programs were used for the electroencephalogram data analysis. Data were filtered offline using a bandpass Bessel (8-pole) 0.04- to 56-Hz filter. Root mean square of the electroencephalogram was computed for 2.56-second segments.

All EEG studies involved a single 60-min general anesthetic exposure to sevoflurane (fig. 1).

We have previously verified that no obvious differences in electroencephalographic activity were detected when electroencephalogram electrode implantation was done either immediately prior or 1–2 days before the start of electroencephalogram recording [17].

Electroencephalogram patterns characterized by an amplitude of at least three times higher than baseline and rhythmic (>2 Hz) activity that lasted for at least 3 s and abruptly reverted to baseline were defined as seizure-like electroencephalogram patterns. In most cases, these patterns started as high frequency-low amplitude activity that developed to increased amplitude and decreased frequency and then abruptly reverted to baseline activity. All parameters for EEG seizures, such as the total duration, number of episodes, and average episode duration, were calculated for the entire 60-min period of sevoflurane exposure (fig. 1). For example, the average episode duration of EEG seizures was calculated as the total duration of EEG seizures during 60 min of anesthesia with sevoflurane divided by the number of episodes of EEG seizures during 60 min of anesthesia with sevoflurane. Continuous isolated spikes that appeared in electroencephalograms recorded in anesthetized rat pups were not considered as EEG seizures and were analyzed separately. The EEG spike activity during 60 min of exposure to sevoflurane was analyzed during three 5-min periods: (1) early during sevoflurane exposure (5–10 min of anesthesia); (2) in the middle of sevoflurane exposure (30–35 min of anesthesia with sevoflurane), and (3) at the end of exposure (55–60 min of exposure to sevoflurane) (fig. 1). The investigators analyzing the EEGs were blinded to the experimental conditions, and all EEGs were reviewed by 3 independent reviewers. About 5% of animals exhibited episode(s) of seizure-like electroencephalogram patterns before the start of anesthesia and were not included in the data anal-

ysis. Histopathological analysis linked most of these seizures to brain injuries during surgery for the EEG electrode implantation.

Measurement of Serum Corticosterone and Estradiol

Serum corticosterone and estradiol were measured using commercial ELISA kits (Cayman Chemical Company, Ann Arbor, Mich., USA) following the manufacturer's instructions.

Slice Electrophysiology

Voltage clamp recordings of membrane ionic currents were conducted by using an Axopatch 200B amplifier (Axon Instruments Inc.) as previously described [1]. In brief, brain hippocampal slices were prepared from P4–P6 rats. The brain was removed after decapitation and was placed into ice-cold sucrose buffer containing (in millimoles): 254 sucrose, 10 D-glucose, 26 NaHCO₃, 2 CaCl₂, 2 MgSO₄, 3 KCl, and 1.25 NaH₂PO₄, saturated with 95% O₂/5% CO₂, at pH 7.4. Transverse hippocampal slices (300 μ m thick) were cut with a VT 1000S microtome (Leica Microsystems Inc., Buffalo Grove, Ill., USA). Slices were transferred immediately into a holding chamber and were incubated at 32–33°C for a 30-min recovery period in a mixture of 50% sucrose saline and 50% artificial cerebrospinal fluid (aCSF) containing (in millimoles): 128 NaCl, 10 D-glucose, 26 NaHCO₃, 2 CaCl₂, 2 MgSO₄, 3 KCl, and 1.25 NaH₂PO₄. Slices were then placed on a nylon mesh, submerged in normal aCSF bubbled continuously with 95% O₂/5% CO₂, and maintained at room temperature (~21–24°C) until whole-cell patch clamp recording, typically within 0.5–5 h.

The slices were transferred to a submersion-type recording chamber (Warner Instruments, Hamden, Conn., USA) on a Burleigh Gibraltar fixed-stage system (Burleigh Instruments, Fisher, N.Y., USA), secured beneath a nylon harp, and perfused with aCSF heated to 30–33°C with an inline heater (Warner SC-20) at a rate of 2–3 ml/min. CA1 pyramidal cells were identified visually by using a microscope (Leica DM LFS, Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) equipped with a $\times 40$ water immersion objective coupled with an infrared differential interference contrast camera system. Whole-cell patch clamp recordings were established using an Axopatch 200B amplifier (Axon Instruments). Membrane current and potential signals were digitized and analyzed with Digidata 1322A (Molecular Devices, Sunnyvale, Calif., USA) and pClamp 10.0 systems (Molecular Devices). Patch pipettes of approx. 5 M Ω were pulled with a P-1000 puller (Sutter Instruments, Novato, Calif., USA). The pipette solution had the following composition (in millimoles), unless stated otherwise: 140 KCl, 0.1 CaCl₂, 5 EGTA, 10 HEPES, 4 ATP-Mg²⁺, 0.4 GTP-2Na⁺, 1 QX314 (lidocaine N-ethyl bromide), pH 7.2 and 290 mOsm. The diffusion potential (liquid junction potential) was 4 mV, calculated by Clampex software (Molecular Devices). QX314 was added to the pipette solution to block the GABA_BR-mediated currents and to prevent the generation of Na⁺-dependent action potentials. Under these conditions, mPSCs were acquired in aCSF containing tetrodotoxin (1 μ M) at a holding potential of –70 mV. To record GABA_AR-mediated mPSCs, glutamate receptor antagonists DNQX (6,7-dinitroquinoxaline-2,3-dione, 20 μ M) and AP5 (DL-2-amino-5-phosphonopentanoic acid, 20 μ M) were added to aCSF. Drugs were administered by bath application. Estradiol (1 μ M) was given after 10 min of baseline recording, and application of picrotoxin (100 μ M) was initiated at 30 min from the start of the recording (20 min after initiation of estradiol application). Synaptic currents were collected for

5 min for each experimental condition. Access resistance (<25 M Ω) was regularly monitored during recordings, and cells were rejected if the resistance changed by >15% during the experiment. If the access resistance increased during the course of the experiment and caused significant reductions in the synaptic current amplitudes, efforts were made to improve access (such as applying additional suction or a slight positive pressure); if this failed, the experiment was discontinued. Synaptic currents were filtered at 2 kHz and were digitized at 10 kHz using Digidata 1322A (Molecular Devices). Offline data analysis was performed using the MiniAnalysis software (version 6.0.7; Synaptosoft, Decatur, Ga., USA) by evaluating the last 3-min interval of each experimental condition. An amplitude threshold of 10 pA was used for automatic detection. Events were then visually screened to eliminate false detections.

Measurement of Latency to LORR

The rat pup was placed in the anesthetic prefilled chamber, and the time during which the rat pup lost the response to slow rotating-like movements of the chamber was recorded with a stopwatch. This time was considered the time needed for sevoflurane to induce LORR.

Drugs

Sevoflurane was manufactured by Fushimi-machi (Osaka, Japan). RU 28318 was purchased from R&D Systems Inc. (Minneapolis, Minn., USA), bumetanide (Ben Venue Laboratories Inc., Bedford, Ohio, USA) was purchased from Bedford Laboratories™ (Bedford, Ohio, USA), and formestane, β -estradiol and ICI 182780 were acquired from Sigma-Aldrich (St. Louis, Mo., USA).

Statistical Analysis

SigmaPlot 12.5 software (Systat Software Inc., Point Richmond, Calif., USA) was used for statistical analyses. Single comparisons were tested using the t test or paired t test (slice electrophysiology data). Multiple comparisons of electroencephalographic seizure parameters among the treatment groups were analyzed using one-way ANOVA, followed by Fisher's LSD test for pairwise comparisons. Two-way repeated-measures ANOVA was used to analyze the frequency and amplitude of spikes (fig. 2d). The normality assumption test was performed using the Shapiro-Wilk test with a p value set at 0.05. All comparisons were run as two-tailed tests. All numerical values are expressed as the mean $\pm$ SE. A $p \leq 0.05$ was considered significant. The sample sizes in this study were based on previous experience with the same experimental techniques [2, 13, 16, 17].

Results

Sevoflurane Causes Similar Hyperexcitation Events in Electroencephalograms of Male and Female Rat Pups

In agreement with our previous findings, anesthesia of P4–P6 rats with sevoflurane was associated with episodes of electroencephalographic seizures (fig. 2a–c). The analysis of electroencephalographic recordings revealed no statistically significant differences in sevoflurane-caused

Fig. 2. Episodes of seizures and persistent regular spike activity in the electroencephalograms in P4–P6 rats during anesthesia with sevoflurane. **a** Root mean square (RMS) of the electroencephalogram of a P4 rat before and during exposure to sevoflurane. Horizontal lines mark the occurrence of episodes of electroencephalographic seizures (1) and spike electroencephalographic activity (2); the corresponding sections of the electroencephalogram are shown at an expanded time scale in **b**. **b** Examples of an episode of electroencephalographic seizures (1) and electroencephalographic spike activity (2) in a P4 rat during exposure to sevoflurane. **c** Histograms showing parameters of electroencephalographic seizures during 60 min of exposure to sevoflurane of P4–P6 rats ($n = 21$). **d** Histograms showing the frequency and amplitude of electroencephalogram spike activities in P4–P6 rats during exposure to sevoflurane. EEG spike activity during 60 min of exposure to sevoflurane was analyzed during three 5-min periods: (1) early during anesthesia (5–10 min of anesthesia); (2) in the middle of sevoflurane exposure (30–35 min of anesthesia with sevoflurane), and (3) at the end of exposure (55–60 min of exposure to sevoflurane; see illustration of the experimental protocols in fig. 1). The spike amplitude was measured as the difference between the baseline values and the peak values either in positive or negative directions.

seizures between male and female rat pups, although all parameters were slightly greater for male rats. The number of episodes of electroencephalographic seizures, the duration of a single episode and the total duration of seizures during 1 h of anesthesia were 4.8 ± 1.263 and 3.727 ± 1.028 ($t_{19} = 0.664$; $p = 0.515$), 6.1 ± 1.431 and 5.618 ± 1.106 s ($t_{19} = 0.269$; $p = 0.791$), and 40.6 ± 10.94 and 29.09 ± 8.13 s ($t_{19} = 0.855$; $p = 0.403$) in male ($n = 10$) and female ($n = 11$) rat pups, respectively. In addition to episodes of electroencephalographic seizures, regular persistent spikes appeared in the electroencephalograms of 7 male and 8 female animals (fig. 2b, d). The spike activity in the sevoflurane-anesthetized animals frequently appeared in clusters consisting of several individual spikes. Two-way

repeated-measures ANOVA of the frequency of spikes in the electroencephalograms of sevoflurane-anesthetized male and female rats sampled during three 5-min intervals of a single anesthetic (at 5–10, 30–35 and 55–60 min of sevoflurane exposure) did not detect significant differences for gender ($F_{1,19} = 0.0334$; $p = 0.857$) or sampling period ($F_{2,19} = 0.319$; $p = 0.729$). The respective values for the amplitude of spikes were also similar (gender: $F_{1,19} = 0.125$; $p = 0.728$; sampling period: $F_{2,19} = 2.962$; $p = 0.065$). Since electroencephalographic seizures and spikes and all following modulations of these activities by study agents were similar in male and female rats, all subsequent data were analyzed and presented as a combination of results obtained from male and female rat pups.

Fig. 3. Sevoflurane-caused electroencephalographic seizures, but not spikes, were diminished by the mineralocorticoid receptor antagonist RU 28318, the estradiol receptor antagonist ICI 182780, and the estradiol synthesis inhibitor formestane. **a** Histograms showing parameters of electroencephalographic seizures in P4–P6 rats of both genders exposed to sevoflurane for 60 min that received as pretreatment DMSO or saline (sevoflurane group, $n = 21$), RU 28318 ($n = 9$), ICI 182780 ($n = 11$) or formestane ($n = 10$). Animals in the sevoflurane group are the same as in figure 2. * $p < 0.05$ versus the sevoflurane group. **b** Examples of electroencephalogram spike activity in sevoflurane-anesthetized P4–P6 rats that received RU 28318, ICI 182780 or formestane 30 min prior to the initiation of anesthesia with sevoflurane. **c** Histograms showing the frequencies of electroencephalogram spike activities in rats exposed to different treatments (the same animals as in **a**). The frequencies are presented as average values measured during three 5-min-long time periods of exposure to sevoflurane for 60 min: 5–10, 30–35 and 55–60 min since initiation of anesthesia with sevoflurane. **d** Histograms showing serum levels of corticosterone in P4–P6 rats of three treatment groups: control, not exposed to sevoflurane ($n = 6$), anesthetized with sevoflurane ($n = 4$) and pretreated with ICI 182780 prior to exposure to sevoflurane ($n = 4$). Rats in the sevoflurane only group received DMSO as a vehicle. Each group consisted of equal numbers of male and female rats. * $p < 0.001$ versus the control group.

Modulation of Hyperexcitatory Electroencephalographic Effects of Sevoflurane by Agents Affecting Steroid Physiology

The total duration ($F_{4,53} = 6.494$; $p < 0.001$), the number of episodes ($F_{4,53} = 5.424$; $p < 0.001$) and the duration of a single episode ($F_{3,46} = 5.383$; $p = 0.003$) of electroencephalographic seizures caused by sevoflurane were significantly diminished by the estradiol receptor antagonist, ICI 182780, and the inhibitor of estradiol synthesis formestane administered prior to the initiation of anesthesia with sevoflurane (fig. 3a). RU 28318 did not significantly reduce the duration of a single episode of sevo-

flurane-caused seizures ($p = 0.364$; fig. 3a). RU 28318 reduced the number of episodes to 1.667 ± 0.601 ($p = 0.014$) and the total duration of seizures to 12.00 ± 5.467 s ($p = 0.008$), respectively. The reductions of the sevoflurane-caused electroencephalographic seizures caused by ICI 182780 and formestane were even more prominent (fig. 3a). The number of episodes was reduced to 0.909 ± 0.392 ($p < 0.001$) and 0.4 ± 0.221 ($p < 0.001$), and the total duration of seizures was shortened to 3.364 ± 1.41 s ($p < 0.001$) and 1.9 ± 1.069 s ($p < 0.001$) in the ICI 182780- and the formestane-pretreated rats, respectively. These agents, with the exception of the mineralocorticoid receptor an-

Fig. 4. Exogenous estradiol increased sevoflurane-caused seizures, spikes and serum levels of corticosterone. The estradiol-enhanced seizures were depressed by ICI 182780, the NKCC1 inhibitor, bumetanide and RU 28318. All these agents, except for RU 28318, diminished the estradiol-enhanced spikes. **a** Histograms showing parameters of electroencephalographic seizures in P4–P6 rats of both genders exposed to sevoflurane for 60 min that received as pretreatment DMSO or saline (sevoflurane group, $n = 21$), estradiol ($n = 15$), the NKCC1 inhibitor, bumetanide ($n = 8$), RU 28318 ($n = 8$) or ICI 182780 ($n = 6$). Animals in the sevoflurane group are the same as in figures 2 and 3. **b** Examples of electroencephalographic seizures [estradiol (E) + sevoflurane (S)] and spikes in electroencephalograms of P4–P6 rats pretreated with estradiol plus bumetanide (E + bumetanide + S), estradiol plus RU 28318 (E + RU 28318 + S) and estradiol plus ICI 182780 (E + ICI 182780 + S) prior to exposure to sevoflurane. **c** Histograms showing the frequencies of electroencephalogram spike activities in rats exposed to different treatments (the same animals as in **a**) prior to exposure to sevoflurane. The frequencies are presented as average values during three 5-min-long time periods of exposure to sevoflurane for 60 min: 5–10, 30–35 and 55–60 min since the initiation of anesthesia with sevoflurane. **d** Histograms showing serum levels of corticosterone in P4–P6 rats anesthetized with sevoflurane ($n = 4$) and pretreated with estradiol prior to exposure to sevoflurane ($n = 5$). Rats in the sevoflurane only group, which is the same group as in figure 3d, received DMSO as a vehicle. Each group consisted of equal numbers of male and female rats. **a, c, d** $p < 0.05$ versus the sevoflurane group; $* p < 0.05$ versus the estradiol plus sevoflurane group.

tagonist RU 28318, tended to reduce the frequency of sevoflurane-caused spikes in the electroencephalograms, but no statistical significance was achieved ($F_{4,45} = 0.878$; $p = 0.485$; fig. 3b, c).

In agreement with the depressant effects of the mineralocorticoid receptor antagonist RU 28318 on seizures caused by sevoflurane, anesthesia with sevoflurane significantly elevated the serum levels of corticosterone, an effect that was not reduced by pretreatment with the estradiol receptor antagonist ICI 182780 ($F_{2,11} = 16.350$;

$p < 0.001$; fig. 3d). We were not able to detect estradiol in serum using an ELISA kit (Cayman Chemical Company).

Exogenous Estradiol Potentiates Hyperexcitatory Effects of Sevoflurane and Serum Levels of Corticosterone

Exogenous estradiol, administered prior to the initiation of anesthesia with sevoflurane, increased the number of episodes of seizures to 8.667 ± 2.173 ($t_{34} = -2.154$; $p =$

Fig. 5. Estradiol increased mPSCs in P4–P6 rat hippocampal CA1 neurons. **a** Illustration of the experimental protocol. Horizontal lines indicate time of treatment applications. **b** Examples of mPSC recordings from a P5 hippocampal CA1 neuron before the administration of estradiol (baseline activity, gray), in the presence of 1 μ M estradiol (dark red) and in the presence of estradiol plus 100 μ M picrotoxin (black). **c–h** Histograms showing the frequency, 10–90% rise, amplitude, area under the curve, and 90–37% decay of mPSCs before the application of estradiol (gray) and in the presence of estradiol (dark red). * $p < 0.005$ versus baseline, $n = 7$. **e** Average mPSCs during the last 3 min of baseline recording (gray) and in the presence of estradiol (17–20 min after the start of administration of estradiol, dark red). **i–l** Estradiol did not affect mPSCs when it was applied in combination with the estradiol receptor antagonist, ICI 182780 (1 μ M). **i** Average mPSCs during the last 3 min of baseline recording (gray) and in the presence of estradiol (1 μ M) plus ICI 182780 (27–30 min period, dark pink). **j–l** Histograms showing the amplitude, frequency and area under the curve of mPSCs during the same periods as in **i** ($n = 3$). Data in the figure are normalized to baseline. Pairwise statistical comparisons were done on the raw data (see text).

0.0384), the total duration of seizures to 98.00 ± 23.650 s ($t_{34} = -2.965$; $p = 0.00551$) and the duration of a single episode to 10.450 ± 1.771 s ($t_{34} = -2.537$; $p = 0.0159$; fig. 4a, b). Estradiol similarly increased the number of episodes of seizures ($t_{13} = 0.768$; $p = 0.457$), the total duration of seizures ($t_{13} = 0.757$; $p = 0.463$) and the duration of a single episode of seizures ($t_{13} = 0.793$; $p = 0.442$) in male and female rat pups anesthetized with sevoflurane. The frequency of spikes was also increased ($t_{34} = -2.559$; $p = 0.0151$; fig. 4b, c). In contrast to animals exposed to sevoflurane only, all animals pretreated with estradiol prior to the initiation of anesthesia with sevoflurane exhibited spike activity in their electroencephalograms.

The total duration of electroencephalographic seizures, enhanced by exogenous estradiol, was diminished by ICI 182780 (4.3 ± 4.333 s; $p = 0.002$) and RU 28318 (19.5 ± 11.087 s; $p = 0.004$), and was almost completely eliminated by the NKCC1 inhibitor bumetanide (1.4 ± 1.375 s; $p < 0.001$; fig. 4a). Likewise, ICI 182780 and bumetanide, but not RU 28318, significantly depressed the frequency of spike activity enhanced by estradiol in sevoflurane-anesthetized animals ($F_{3,35} = 10.309$; $p < 0.001$; fig. 4b, c).

In agreement with the increase in seizure-like EEG activity by exogenous estradiol in sevoflurane-anesthetized animals and the depressant effect of the mineralocorticoid receptor antagonist RU 28318 on these estradiol-en-

Fig. 6. Estradiol shortens and ICI 182780 and formestane lengthen the time needed for sevoflurane to induce LORR in P4–P6 rats. Histogram showing the time to LORR caused by sevoflurane (3.5%) in P4–P6 rats that received DMSO (sevoflurane group, $n = 5$), estradiol ($n = 5$), ICI 182780 ($n = 6$) or formestane ($n = 5$) 30 min before a rat pup was placed in a thermostated chamber filled with 3.5% sevoflurane. * $p < 0.05$ versus the sevoflurane group.

hanced seizures, exogenous estradiol further increased serum levels of corticosterone ($t_7 = 2.814$; $p = 0.026$; fig. 4d), the predominant agonist for mineralocorticoid receptors in the central nervous system.

Estradiol Enhances GABA_AR-Mediated Synaptic Responses in Hippocampal CA1 Neurons

mPSCs were recorded from hippocampal CA1 neurons in freshly prepared P4–P6 rat brain slices superfused with aCSF containing antagonists of glutamate AMPA/kainate and NMDA receptors. Estradiol was added to the superfusing aCSF after establishing baseline activity for 10 min (fig. 5a). The whole-cell recordings of mPSCs revealed that estradiol enhanced mPSC activity (fig. 5a–h), an effect that required more than 10 min to develop. For comparison, the GABA_AR antagonist picrotoxin, added to aCSF at the end of the experiment, almost completely depressed the mPSC activity 2–3 min after the start of its application (fig. 5b). Picrotoxin blocked the estradiol-enhanced mPSCs in all cells studied, suggesting that estradiol enhanced GABA_AR-mediated mPSCs. A comparison of the

mPSC activities during the final 3 min of baseline recordings (7–10 min) and estradiol applications (27–30 min) revealed that estradiol enhanced mPSC amplitude from 19.62 ± 1.96 to 27.22 ± 2.4 pA ($t_6 = -3.381$; $p = 0.00816$; fig. 5b, f), mPSC 10–90% rise time from 3.19 ± 0.39 to 2.40 ± 0.39 ms ($t_6 = -3.747$; $p = 0.00954$; fig. 5d), and mPSC area under the curve from 514.48 ± 76.37 to 680.35 ± 83.87 pAms ($t_6 = -2.854$; $p = 0.029$; fig. 5g). The mPSC frequency ($t_6 = -0.590$; $p = 0.577$; fig. 5c) and 90–37% decay time ($t_6 = -0.305$; $p = 0.771$; fig. 5h) were not affected by estradiol. Application of estradiol in combination with the estradiol receptor antagonist ICI 182780 resulted in the prevention of the effect of estradiol on mPSCs (fig. 5j–l).

Exogenous Estradiol Shortens while ICI 182780 and Formestane Lengthen the Latency to LORR Induced by Sevoflurane

Most of the cortical hyperexcitatory effects of endogenous and exogenous estradiol observed in this study are consistent with its action through the modulation of GABAergic transmission. If estradiol is involved in the mediation of the effects of sevoflurane by enhancing GABA_AR activity, then it should enhance the inhibitory effects of sevoflurane in neonatal rats, mediated by caudal neuronal circuitries in which GABA_AR signaling has already matured from excitatory to inhibitory. For this purpose, the effects of exogenous estradiol, the antagonist of estrogen receptor ICI 182780 and the inhibitor of estradiol synthesis formestane were tested on the latency of sevoflurane to induce LORR, which is predominantly mediated by midbrain neuronal networks. We found that estradiol reduced, and ICI 182780 and formestane increased the time needed for sevoflurane to induce LORR ($F_{3,17} = 16.693$; $p < 0.001$; fig. 6).

Discussion

The results of this study demonstrate that sevoflurane increases serum levels of corticosterone and causes electroencephalographic seizures and spikes that are depressed by corticosterone and estrogen receptor antagonists, by an inhibitor of estrogen synthesis, and are facilitated by exogenous estradiol. These findings suggest an involvement of steroids in the mediation of the hyperexcitatory cortical effects of sevoflurane in neonatal rats. Furthermore, the results of this study provide evidence for the involvement of endogenous estradiol in the induction of general anesthesia by sevoflurane in neonatal rats by participating in sevoflurane-induced LORR.

In addition to the previously reported depression of electroencephalographic seizures by bumetanide [13, 16, 17], an agent that shifts GABA_A-mediated signaling in immature neurons from excitatory to inhibitory [18, 19], the current findings indicate that sevoflurane-caused seizures could also be diminished by mineralocorticoid and estradiol receptor antagonists and an inhibitor of estradiol synthesis. Exogenous estradiol increased sevoflurane-caused seizures, an effect that was reduced by the same treatments that led to the depression of electroencephalographic seizures caused by sevoflurane alone. These findings suggest an important role of steroids, in general, and estradiol, in particular, in the developmental hyperexcitation effects caused by sevoflurane in neonatal rats. In agreement with this role, estradiol is known to exert profound and diverse effects on the developing brain [20, 21]. Estradiol is at its highest levels during the perinatal period, and it is measured at the same levels in the male and female neonatal rat cortex [5, 6], supporting that similar potencies of sevoflurane cause cortical hyperexcitation in male and female pups found in this study.

The depressant effects of bumetanide on sevoflurane-caused seizures [13, 16, 17] and estradiol-facilitated sevoflurane-caused seizures point to a role of GABA_A-mediated excitation in these reported effects of sevoflurane and estradiol. This possibility is further supported by our finding that the acute application of estradiol to rat hippocampal slices *in vitro* resulted in increased GABA_A-mediated responses. Known effects of estradiol on GABA_A-mediated signaling include an estradiol-mediated increase in the concentration of GABA-synthesizing enzymes and GABA_A proteins in the olfactory bulb of the male rats [8], as well as in the hippocampal activity of GABA_A in P7–P13 rats [9]. Furthermore, estradiol may enhance GABA_A-mediated depolarization by upregulating NKCC1 transporter activity in neonatal hypothalamic neurons [10]. Estradiol-caused elevation of intracellular calcium in immature neurons through enhancement of the depolarizing action of GABA is thought to be a primary reason why in neonatal rats estradiol exacerbates hippocampal cell damage in the presence of a GABA_A agonist [22]. A role for GABAergic mechanisms in the action of estradiol is also supported by our findings that exogenous estradiol shortened and the estrogen receptor antagonist and inhibitor of synthesis of estradiol lengthened the time needed for sevoflurane to induce LORR. The righting reflex is predominantly mediated by neuronal circuits in the midbrain [14, 15]. In the P4–P6 rats, GABA_A-mediated signaling is predominantly excitatory in rostral regions such as cortical and hippocam-

pal neurons but becomes increasingly inhibitory in more caudal regions [18, 19]. The estradiol-, ICI 182780-, and formestane-altered times needed for sevoflurane to cause LORR support that estradiol may act not only through upregulation of NKCC1 activity, but also through enhancement of neuronal inhibition, likely by increasing GABAergic activity.

Sevoflurane and exogenous estradiol increased serum levels of corticosterone, while the electroencephalographic seizures caused by these agents were depressed by the corticosteroid receptor antagonist, RU 28318, indicating that corticosterone may be involved in the mediation of the electroencephalographic seizures, as we previously demonstrated for propofol-caused seizures in neonatal rats [2]. The known ability of corticosterone to potentiate excitatory glutamatergic activity [23, 24] provides a mechanistic basis for corticosterone involvement in electroencephalographic seizures caused by sevoflurane or propofol [2] in neonatal rats. It is plausible that the production and release of corticosterone that contributes to the electroencephalographic effects of sevoflurane is stimulated by sevoflurane alone, and not facilitated by physiological levels of estradiol, since the estradiol receptor antagonist ICI 182780 did not decrease the rise in serum levels of corticosterone caused by sevoflurane. This suggests that the main effect of endogenous estradiol during anesthesia with sevoflurane is mediated through enhancement of GABAergic activity.

Previously, Wójtowicz et al. [9] reported that long-term treatment with exogenous estradiol was needed to induce an increase in the amplitude and frequency of mPSCs and the amplitude of GABAergic tonic current in CA1 pyramidal cells of P7–P13 rats, while estradiol did not affect GABA_A-mediated currents in rats older than P22. Also, up to 24 h were required to detect an increase in the NKCC1 activity in hypothalamic neurons after the administration of estradiol to P0 rats [25]. We observed increases in GABA_A-mediated mPSCs in hippocampal CA1 pyramidal cells of P4–P6 rats within 10–20 min of estradiol applications. These effects of estradiol in brain slices are in agreement with our *in vivo* findings demonstrating that the electroencephalographic effects of estradiol, the antagonist of its receptors and the inhibitor of its synthesis can be detected within 30 min after their administration, the time when anesthesia with sevoflurane was initiated. The time frame of the effects of estradiol observed in this study is consistent with rapid estrogenic neuronal effects that could also be linked to the regulation of behavior and cognition [26, 27]. These effects may occur within seconds to minutes, but generally within 1 h,

and can subsequently regulate nuclear events [28]. It has been discussed that estrogens underlying these rapid actions are primarily synthesized de novo in the brain [29], and brain levels of estradiol are higher than those in the circulation [30]. This may be one plausible explanation why we were not able to detect an increase in serum levels of estradiol in neonatal rats anesthetized with sevoflurane, an important limitation of the current study. To test the possibility whether brain-produced estradiol is involved in mediating the effects of sevoflurane, we have been working to develop an assay to measure neurosteroid and estradiol levels in the neonatal rat brain using the liquid chromatography-mass spectrometry technique.

GABA_AR-mediated depolarization, but not activation of corticosteroid receptors, is likely responsible for the persistent spike activity in the electroencephalograms since spike activity was depressed by bumetanide and further enhanced by exogenous estradiol, but was insensitive to pretreatment with RU 28318. We previously reported that RU 28318 did not affect spike activity caused by propofol [2]. Weak effects of the estrogen receptor antagonist and inhibitor of estradiol synthesis on spike activity in animals anesthetized with sevoflurane suggest that direct GABAergic effects of sevoflurane may be sufficient to support this spike activity. Electroencephalographic sei-

zures and persistent spike activity suggest an increase in the excitation/inhibition ratio in neonatal rats over the entire period of exposure to sevoflurane.

In summary, the results of this study, in combination with previously published data, demonstrate important roles of steroids, particularly estradiol, in the central nervous system effects of sevoflurane in neonatal rats. Estradiol may be involved in both developmental hyperexcitatory effects of sevoflurane and in sevoflurane's inhibitory LORR effect.

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