Roles of Aldosterone and Oxytocin in Abnormalities Caused by Sevoflurane Anesthesia in Neonatal Rats

Wengang Cao, M.D.,* Christopher Pavlinec, B.S.,† Nikolaus Gravenstein, M.D.,‡ Christoph N. Seubert, M.D., Ph.D.,§ Anatoly E. Martynyuk, Ph.D., D.Sc.||

ABSTRACT

Background: The authors sought to determine whether subjects with pathophysiological conditions that are characterized by increased concentrations of aldosterone have increased susceptibility to the side effects of neonatal anesthesia with sevoflurane.

Methods: Postnatal day 4–20 (P4–P20) rats were exposed to sevoflurane, 6% and 2.1%, for 3 min and 60–360 min, respectively. Exogenous aldosterone was administered to imitate pathophysiological conditions with increased concentrations of aldosterone.

Results: Six hours of anesthesia with sevoflurane on P4–P5 rats resulted in a more than 30-fold increase in serum concentrations of aldosterone (7.02 ± 1.61 ng/dl vs. 263.75 ± 22.31 ng/dl, mean ± SE, n = 5–6) and reduced prepulse inhibition of the acoustic startle response (F(2,37) = 5.66, P < 0.001). Administration of exogenous aldosterone during anesthesia with sevoflurane enhanced seizure-like electroencephalogram patterns in neonatal rats (48.25 ± 15.91 s vs. 222.00 ± 53.87 s, mean ± SE, n = 4) but did not affect electroencephalographic activity in older rats. Exogenous aldosterone increased activation of caspase-3 (F(3,38) = 11.02, P < 0.001) and disruption of prepulse inhibition of startle (F(3,40) = 6.36; P = 0.001) caused by sevoflurane. Intracerebral administration of oxytocin receptor agonists resulted in depressed seizure-like electroencephalogram patterns (F(2,17) = 6.37, P = 0.009), reduced activation of caspase-3 (t(11) = 2.83, P = 0.016), and disruption of prepulse inhibition of startle (t(7) = −2.9; P = 0.023) caused by sevoflurane.

What We Already Know About This Topic
• Sevoflurane is known to cause adverse effects on the brains of neonatal animals
• Aldosterone levels are elevated in newborns

What This Article Tells Us That Is New
• In a neonatal rat model, sevoflurane increased serum levels of aldosterone
• Exogenous aldosterone exacerbated the neurodevelopmental effects of sevoflurane

Conclusions: These results suggest that adverse developmental effects of neonatal anesthesia with sevoflurane may involve both central and peripheral actions of the anesthetic. Subjects with increased concentrations of aldosterone may be more vulnerable, whereas intracerebral oxytocin receptor agonists may be neuroprotective.

MECHANISMS mediating the adverse neurodevelopmental actions of general anesthetics in the early stages after birth1–5 and by extension conditions that may specifically affect the severity of these side effects are essentially unknown. We recently reported that in neonatal rats the Na⁺–K⁺–2Cl⁻ cotransporter (NKCC1) inhibitor bumetanide diminished sevoflurane-caused, seizure-like electroencephalogram patterns and decreased a biomarker of apoptosis, activated caspase-3.4 High expression of NKCC1 in late embryonic and early neonatal cortical and hippocampal neurons is responsible for the increased concentrations of intracellular Cl⁻ that provide the basis for a depolarizing and excitatory action of γ-aminobutyric acid (GABA) through stimulation of the GABA receptor type A (GABA_A) receptors (GABAAR).6 Therefore, the results of the experiments with bumetanide suggest that increased excitability of neonatal neurons, which is enhanced by sevoflurane (an action that includes but is not necessarily limited to stimulation of GABAAR activity), underlies the anesthetic-induced, seizure-like electroencephalogram patterns and activation of caspase-3.4 The notion of deleterious effects of enhanced excitation in the developing brain also is supported by findings that oxytocin, which inhibits NKCC1 and neuronal activity, exerted neuroprotective effects on the fetal brain.7 NKCC1-GABAAR-mediated depolarizing signaling plays an important regulatory role in the adrenal gland.8,9 Aldosterone, which is produced by adrenocortical cells, is known
to cause a number of adverse effects, including oxidative stress, inflammation, apoptosis, and cognitive impairments.\textsuperscript{10–14} Importantly, the action of aldosterone in the brain may be age dependent, with more pronounced effects in neonatal brain than in adult brain because of higher expression of 11-\(\beta\)-hydroxysteroid dehydrogenase 2 in early postnatal brain.\textsuperscript{15,16} We tested whether anesthesia of neonatal rats with sevoflurane leads to increased concentrations of aldosterone and whether neonates with pathophysiological conditions that are characterized by increased concentrations of aldosterone that may be more susceptible to the side effects of sevoflurane. These pathophysiological conditions, which may include premature birth, sepsis, and cardiovascular and other disorders,\textsuperscript{14,17–19} were imitated by administering exogenous aldosterone. The changes in electroencephalographic activity, concentrations of activated caspase-3, and prepulse inhibition (PPI) of the acoustic startle response in rats that were exposed to sevoflurane anesthesia during the early postnatal period were investigated. The effects of exogenous agonists of oxytocin receptors also were assessed, given the inhibitory effect of activation of oxytocin receptors on NKCC1 activity, activity of neonatal neurons, and plasma aldosterone concentrations.\textsuperscript{7,20}

The PPI of startle, the reduction of the startle response when the startle stimulus is preceded by a subthreshold sensory stimulus (sensorimotor gating), is mediated by multiple forebrain structures, including the nucleus accumbens, hippocampus, amygdala, striatum, and prefrontal cortex.\textsuperscript{21} Various neurotransmitter and hormonal systems are involved in modulation of the PPI of startle. It is assumed that the normal filtering of unnecessary sensory, cognitive, and motor information is essential for mental and behavioral integration.\textsuperscript{22} Reduction of the PPI may reflect impairment in this process, which is evident in many neurocognitive disorders, most notably in schizophrenia.\textsuperscript{16} Disruption of the PPI can be induced by many developmental and pharmacologic manipulations or by stressful conditions.\textsuperscript{23–25} Given that the PPI measurements can be easily replicated in various species, including humans, investigation of the effects of neonatal anesthesia on PPI of the startle response may facilitate translational research in developmental anesthesia toxicity, an area in which a link between animal and human studies has yet to be established.

Materials and Methods

Animals

All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee (Gainesville, Florida). Sprague-Dawley rats of both genders were studied. To control for litter variability, we used several pups from each litter for different treatment conditions. At the beginning of each experiment, the younger pups were determined to be well nourished, as judged by their stomachs being full of milk (detectable through the transparent abdominal wall). Different sets of animals were used in each given experiment. Each animal was studied in only one experiment.

Anesthesia and Electroencephalogram Recording

To determine the effects of sevoflurane on cortical activity, rat pups ranging from postnatal day 4 to 20 (P4–P20) were instrumented for electroencephalogram recording and offline electroencephalogram analysis, as detailed previously.\textsuperscript{4} In brief, four electrodes of the head mounts of the electroencephalogram or electromyogram system (Pinnacle Technology, Lawrence, KS) were implanted during isoflurane anesthesia (1.6–2.0%). No obvious differences in electroencephalographic activities were observed when the recordings were started either immediately or 1–2 days after surgery. Sevoflurane (Fushimi-machi, Osaka, Japan) anesthesia was induced with sevoflurane (6%) and 1.5 l/min oxygen over 3 min and maintained with sevoflurane (2.1%) and 1.5 l/min oxygen in a thermostated chamber set at +37°C. Onset and offset of anesthesia were monitored via electroencephalogram and by loss and return of righting reflex, respectively. Anesthesia gas monitoring was performed using a calibrated Datex side stream analyzer (Datex-Ohmeda, Helsinki, Finland) that sampled from the interior of the animal chamber. We have shown previously that anesthesia of postnatal rats with sevoflurane (2.1%) for as long as 6 h does not cause hypoxia, hypoventilation, or hypoglycemia.\textsuperscript{4} Electroencephalographic seizure-like patterns were defined as electroencephalogram patterns of high-amplitude rhythmic activity with evolution in frequency or amplitude that were at least three times greater than the baseline activities, lasted for at least 3 s, and abruptly reverted to baseline. Animals that exhibited episode(s) of seizure-like electroencephalogram patterns before the start of anesthesia were not included in the data analysis.

Measurements of Acoustic Startle Response and PPI of Startle

The PPI of startle tests were performed using the SR-Lab startle apparatus and accompanying software (San Diego Instruments, San Diego, CA).

Because the rats received either vehicle or treatment and were anesthetized at P4–P5, they could not be assigned later to treatment groups with similar baseline PPI values between groups that could be determined by performing a “matching” startle session. To avoid the effect of litter variability, we used several pups from three to five different litters for a given PPI experiment (given treatment conditions). Typically, a PPI test for each experimental condition took several months to complete. Testing occurred during the light phase of the light–dark cycle. The sound source was calibrated before every testing session using a digital sound level meter (RadioShack, Fort Worth, TX) with the device placed inside the cylindrical animal enclosure. At the beginning of every testing session, each animal was exposed to a 75 dB white noise (background) for a 5-min acclimation period. The ac-
climination was then followed by a test session consisting of five different types of trials: only 120 dB pulse of 40 ms duration; a 120 dB pulse of 40 ms preceded by a prepulse of 20 ms at 5, 10, and 15 dB above background; and a no-stimulus trial of background noise. The delay between the onset of the prepulse and the onset of the pulse was 100 ms. The trials were presented in pseudorandom order with variable intertrial intervals with an average duration of 15 s. The first four trials and last three trials consisted of 120-dB, pulse-only trials. All five types of trials were presented eight times, each in pseudorandom order after the first four and last three pulse-only trials. The percentage of the PPI for each prepulse intensity was calculated using the following formula: \%PPI = 100 \times [(pulse alone) − (prepulse + pulse)]/ pulse alone. The responses to the first four pulse-only trials, which were used to estimate the startle response, and the last three pulse-only stimuli were not included in the calculations of the percentage of the PPI. Data were collected as average amplitude of the 1,000-ms recording window.

**Determination of Activated Cleaved Caspase-3 Using Western Blot**
The concentrations of activated caspase-3 in the cerebral cortex were determined as described previously.3 Western blot analysis for tissue samples from each animal was done in triplicate and reported as an average.

**Measurement of Serum Aldosterone**
Serum aldosterone was measured by radioimmunoassay using antibody-coated tubes (Siemens, Los Angeles, CA). Trunk blood samples were collected without the addition of anticoagulants. Animals that were exposed to sevoflurane were sacrificed at the end of 6 h of anesthesia. Control rat pups that were not exposed to sevoflurane were anesthetized briefly with isoflurane before decapitation. The assay sensitivity was 1 ng/dl. The intraassay precision was 5% (coefficient of variation) at a mean of 25 ng/dl, and the interassay precision was 6.6% (coefficient of variation) at a mean of 25 ng/dl. The aldosterone measurements were performed by the Wake Forest School of Medicine Hypertension Core Laboratory (Winston-Salem, North Carolina). Investigators there were blinded to the treatment groups.

**Intracerebral Administration of Oxytocin and Carbetocin**
Rats were anesthetized with a mixture of oxygen (1.5 l/min) and isoflurane (3.0%) and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA); anesthesia was maintained for the duration of the surgery using an oxygen-isoflurane (1.6–2%) mixture delivered through a nose cone. The skull was exposed, and a 0.5-mm hole was drilled with a stereotaxic drill (Kopf Instruments) in the cranium dorsal to the left hemisphere. The following stereotaxic coordinates were used (2 mm posterior and 3 mm lateral to the bregma). A Hamilton syringe was stereotactically positioned in the hole and lowered to a depth of 2.7 mm; 1.5 µl saline or saline solution containing 10 mg/ml carbetocin or oxytocin was injected at a rate of 1 µl/min using a stereotaxic injector (Stoelting Co., Wood Dale, IL). The syringe was left in place for 1.5 min before removal and wound closure. The coordinates of the intraventricular cavity were chosen based on our anatomic analysis of the brain of P4–P6 rats, performed after completion of the electroencephalogram measurements. Because we do not have any means to verify the exact area of injection, we refer to these administrations as intracerebral administrations. During surgery, body temperature was maintained between 37.5° and 38.0°C using a heating pad. Rats were treated in a randomized fashion with injections of oxytocin–carbetocin or equal volumes of saline. The electroencephalogram recording was started immediately upon completion of the surgery. In the experiments to investigate the effects of sevoflurane on levels of activated caspase-3 and PPI of startle, anesthesia with sevoflurane was initiated 10 min after the intracerebral administrations.

**Drugs**
Bumetanide (Ben Venen Laboratories, Inc., Bedford, OH) was purchased from Bedford Laboratories (Bedford, OH). Aldosterone was purchased from TRC (Toronto, Ontario, Canada) and from Sigma (St. Louis, MO). Oxytocin was purchased from Sigma. Carbetocin was purchased from Polypeptide Laboratories (Strasbourg, France). Cleaved caspase-3 antibodies were acquired from Cell Signaling (Danvers, MA), and horseradish peroxidase conjugated goat antirabbit and anti-γ-tubulin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Aldosterone (20 mg/kg, administered subcutaneously) was solubilized in dimethyl sulfoxide at 12 mg/ml.

**Statistical Analysis**
Values are reported as mean ± SEM. SigmaStat 3.11 software (Systat Software, Point Richmond, CA) was used for statistical analysis. Single comparisons were tested using the t test, whereas multiple comparisons among groups were analyzed using ANOVA, followed by Holm-Sidak tests. Changes in the PPI of startle for three prepulse intensities in multiple groups were analyzed using repeated measures ANOVA, followed by Holm-Sidak tests. A P < 0.05 was considered significant.

**Results**
Sevoflurane Anesthesia Impairs Sensorimotor Gating Function and Increases Serum Concentrations of Aldosterone
First, we tested whether anesthesia of neonatal rats with sevoflurane causes long-term brain functional effects, beyond the previously reported acute effects, such as seizure-like electroencephalogram patterns and increased concentrations of activated caspase-3 that were alleviated by pretreatment with bumetanide.4 For this purpose, we evaluated the acoustic
Sevoflurane anesthesia on postnatal days 4–5 (P4–P5) impairs sensorimotor gating function and increases serum concentrations of aldosterone. Illustration of the experimental protocols (A). Histogram showing percentage of the prepulse inhibition (PPI) of startle in different treatment groups: control (n = 11), saline + sevoflurane (n = 14), and bumetanide + sevoflurane (n = 15). *P < 0.05 versus nonanesthetized and bumetanide-treated rats. dB = Prepulse intensities in decibels above background (B). Histogram showing percentage of the PPI of startle in nonanesthetized rat pups treated with intraperitoneal bumetanide (5 μmol/kg; n = 13) or an equal volume of saline (n = 10). The different sets of animals were used in the experiments reported in B and C (C). Histograms showing serum concentrations of aldosterone in rats not exposed to sevoflurane anesthesia (saline, n = 5) and in the rats that were anesthetized with sevoflurane and received as pretreatment either saline (n = 6) or bumetanide (n = 4) (D). *P < 0.001 versus aldosterone concentrations in the nonanesthetized rats. $P < 0.014 versus aldosterone concentrations in the anesthetized rats pretreated with bumetanide.

The PPI of startle, two additional groups of animals were studied. Again startle amplitudes between the control and bumetanide-treated animals were comparable (t(21) = -0.71, P = 0.487). Bumetanide in the absence of sevoflurane anesthesia did not affect the PPI of startle (F(2,20) = 0.10 vs. saline, P = 0.902, fig. 1C).

Measurements of serum concentrations of aldosterone at the end of sevoflurane exposure (fig. 1A) showed that animals anesthetized with sevoflurane had significantly increased blood concentrations of aldosterone (F(2,13) = 36.52, P < 0.001 vs. nonanesthetized control, fig. 1D). Serum concentrations of aldosterone were further increased in rats pretreated with bumetanide before sevoflurane anesthesia (P < 0.034 vs. sevoflurane).

**Exogenous Aldosterone Exacerbates the Side Effects of Sevoflurane Anesthesia**

We next studied the effects of exogenous aldosterone on the side effects caused by anesthesia with sevoflurane in neonatal rats. First, we analyzed electroencephalographic activity in P4–P6 rats during sevoflurane anesthesia before and after administration of aldosterone (20 mg/kg, subcutaneously, fig. 2A). Aldosterone increased the number of episodes (t(6) = -2.74, P = 0.034) and total duration of seizure-like electroencephalogram patterns (t(6) = -3.09, P = 0.021) but not the duration of an individual episode (P = 0.162, fig. 2, B and C). A subset of animals was treated with intraperitoneal bumetanide (5 μmol/kg) 60 min after administration; anesthesia with sevoflurane (2.1%) was maintained for another 60 min. Bumetanide failed to diminish aldosterone-enhanced episodes of seizure-like electroencephalogram patterns (P = 0.864, n = 4). In some animals, bumetanide further enhanced the intensity of this excitable electroencephalographic activity recorded after the administration of aldosterone. Importantly, the seizure-like electroencephalogram patterns after administration of aldosterone were more intensive than those seen in the presence of sevoflurane alone. To control for the effects of sevoflurane on electroencephalographic activity during 2 h of anesthesia with sevoflurane, an additional group of P4–P6 animals was studied. There was no significant difference between seizure-like electroencephalogram patterns during the first and second hours of anesthesia with sevoflurane (t(10) = -0.05, P = 0.964, fig. 2, B and C). In contrast to the findings in P4–P6 rats, we did not observe obvious changes in electroencephalographic activity in P17-P20 rats during sevoflurane anesthesia after the administration of aldosterone (n = 8) (fig. 2D).

After we observed the intense seizure-like electroencephalogram patterns upon administration of aldosterone in the experiments described above, we chose a shorter duration of anesthesia (120 min, fig. 3A) to assess the effect of exogenous aldosterone on concentrations of activated caspase-3 in the sevoflurane anesthetized rats. The concentration of activated caspase-3 was significantly increased in the sevoflurane anesthetized rats compared with the nonanesthetized controls (t(13) =
Aldosterone, administered before sevoflurane anesthesia, further increases activation of caspase-3 in rat cerebral cortex and reduction of prepulse inhibition (PPI) of startle. Illustration of the experimental protocols (A). Histogram showing results of the densitometric analysis of cleaved caspase-3 in the cortex tissue from four treatment groups. Rats in the control groups did not undergo anesthesia on postnatal day 4 (P4) but were treated either with (subcutaneous aldosterone, 20 mg/kg, n = 6) or equal volume of vehicle (dimethyl sulfoxide, DMSO; n = 4). The anesthetized rats received as pretreatment aldosterone (n = 11) or vehicle (n = 11). Densities of ␥-tubulin blots from the same tissue sample were taken as 100%. *P < 0.05 versus aldosterone + sevoflurane. Representative Western blot (WB) images of cleaved caspase-3 and ␥-tubulin blots in the cortex tissue of postnatal day 4 (P4) rats are shown below (B). Histograms showing percentage of PPI of startle in four treatment groups: the nonanesthetized rats treated with aldosterone (20 mg/kg, subcutaneous administration, n = 10) or equal volume of vehicle (DMSO, n = 10), and the anesthetized rats that received as pretreatment either aldosterone (n = 15) or vehicle (n = 15). *P < 0.05 versus nonanesthetized rats treated with vehicle, and #P < 0.05 versus nonanesthetized rats treated with aldosterone (C).

Subcutaneous exogenous aldosterone (20 mg/kg) increased caspase-3 activity in the anesthetized rats (F(3,28) = 11.02, P < 0.001) but did not affect such activity in nonanesthetized controls (P = 1.000, fig. 3B).

The long-term effects of exogenous aldosterone again were evaluated by assessing sensorimotor gating. Neither sevoflurane anesthesia for 240 min at P4, nor aldosterone significantly affected the startle response either by themselves or in combination. Values for startle were 14.7 ± 3.1, 13.6 ± 4.2, 15.5 ± 2.6, and 14.7 ± 4.4 for vehicle, aldosterone, vehicle plus sevoflurane, and aldosterone plus sevoflurane, respectively (F(3,46) = 0.54, P = 0.661). Analysis of the PPI of startle by repeated measure ANOVA indicated the expected significant main effect of decreasing startle amplitude with increasing prepulse intensity (F(2,46) = 281.4, P < 0.001) and a significant main effect of treatment (F(3,46) = 6.36; P = 0.001). Multiple pairwise comparisons between treatments showed that, despite the shorter duration of exposure, sevoflurane (fig. 3A) still resulted in significant impairment of the PPI of startle measured at a prepulse intensity of 5 dB in the 24-day-old rats (P = 0.016 vs. control) (fig. 3C). The animals pretreated with aldosterone and anesthetized with sevoflurane had additionally disrupted PPI of startle, which also diminished with increasing prepulse intensity but remained significantly disrupted at all three prepulse intensities (fig. 3C). The rats treated with aldosterone but not exposed to the anesthetic had a PPI not different from nonanesthetized controls.

Fig. 2. Exogenous aldosterone enhances seizure-like electroencephalogram patterns in neonatal but not older rats anesthetized with sevoflurane. Illustration of the experimental protocol (A). Examples of seizure-like electroencephalogram patterns in a postnatal day 6 (P6) rat during the second hour of sevoflurane anesthesia without aldosterone (top) (B). Histograms showing parameters of seizure-like electroencephalogram patterns during sevoflurane (2.1%) anesthesia in another P6 rat after administration of aldosterone (bottom) (C). An example of electroencephalogram recordings from a postnatal day 19 (P19) rat during 2.1% sevoflurane anesthesia before (1) and after (2) administration of aldosterone (D).
from that of controls ($P = 0.231$). Again, there was a significant interaction between treatment and prepulse intensity ($P = 0.027$).

**Oxytocin or Its Synthetic Analog, Carbetocin, Alleviates Side Effects Caused by Sevoflurane Anesthesia in Neonatal Rats**

Rats pretreated with either oxytocin or carbetocin had significantly less seizure-like electroencephalogram patterns compared with rats that received saline as a pretreatment (fig. 4A–C). All parameters of the seizure-like activity that were analyzed, duration ($F_{(2,17)} = 6.37$, $P = 0.009$), number of episodes ($F_{(2,17)} = 7.24$, $P = 0.005$), and duration of an individual episode ($F_{(2,17)} = 5.22$, $P = 0.011$), were diminished significantly in the oxytocin- and carbetocin-pretreated rats compared with the saline-pretreated animals (fig. 4C). Intraperitoneal administration of carbetocin (20 mg/kg) to P5–P9 rats ($n = 10$) did not result in depression of seizure-like electroencephalogram patterns during sevoflurane anesthesia.

To assess effects on neuroapoptosis, two groups of P4 rats were pretreated either with intracerebral carbetocin (1.5 µg in 1.5 µl saline) or equal volumes of saline 10 min before 2 h of anesthesia with sevoflurane. Because of the relatively short half-life of carbetocin (≈40 min) shorter periods of sevoflurane anesthesia were chosen for these experiments. The animals were sacrificed approximately 18 h after completion of anesthesia for determination of the concentrations of activated caspase-3 in the cerebral cortex. There was a significant decrease in concentrations of activated caspase-3 ($t_{(11)} = 2.83$, $P = 0.016$) in the carbetocin-pretreated animals ($20.0 ± 2.0%$ relative to γ-tubulin, $n = 7$) in comparison with those of animals pretreated with saline ($30.4 ± 3.2%$, $n = 6$).

The same treatment paradigms were used in two groups of animals to determine the effect of carbetocin on the PPI of startle (fig. 4D). Again, treatment with carbetocin at P4 did not change the startle response at P24. Startle responses were 13.8 ± 3.8 and 14.8 ± 3.1 for saline- and carbetocin-treated animals, respectively ($t_{(7)} = -0.39$, $P = 0.708$). The PPI of startle response was significantly lower in the saline-treated rats ($t_{(7)} = -2.9$, $P = 0.023$ vs. the carbetocin-treated animals, fig. 4E). This difference was detectable only at a prepulse intensity of 5 dB.

**Discussion**

This study, in combination with previously published works, demonstrates that sevoflurane anesthesia in the early postnatal period of otherwise healthy animals may result in increased concentrations of aldosterone and acute and delayed abnormalities in brain functioning. The side effects caused by sevoflurane were exacerbated by exogenous aldosterone, suggesting that neonates with increased concentrations of aldosterone may be more vulnerable. The sevoflurane-caused side effects could be diminished by intracerebral pretreatment with oxytocin or its synthetic analog carbetocin and systemic pretreatment with bumetanide, suggesting that treatments that decrease excitability of neonatal neurons may make the neonatal brain less susceptible to the adverse effects of sevoflurane.

Anesthesia of neonatal rats with sevoflurane not only causes acute functional (seizure-like electroencephalogram patterns) and cytotoxic (activation of caspase-3 in the cerebral cortex) effects, but also results in long-term developmental abnormalities that could be detected as a reduced PPI of the startle response at a time when the rats achieved postweaning age (fig. 1B). The latter finding is of...
Fig. 5. Illustration of hypothetical pathways or mechanisms mediating the effects of sevoflurane, aldosterone, oxtocin, and bumetanide. + = stimulation; − = inhibition; BBB = blood-brain barrier; EEG = electroencephalogram; GABA_\text{\textsubscript{A}R} = \gamma\text{-aminobutyric acid type A receptors; NKCC1 = the Na\textsuperscript{+}–K\textsuperscript{-}–Cl\textsuperscript{-} cotransporter; PPI = prepulse inhibition of the acoustic startle response. See text for details.}

great interest from a translational research point of view because a crucial question is whether general anesthesia, administered during the early neonatal period, causes long-term developmental effects. Reduced PPI of startle is associated with a number of neuropsychiatric disorders with cognitive dysfunction, such as schizophrenia, autism, obsessive-compulsive disorder, posttraumatic stress disorder, and others.\textsuperscript{21–26,28} For example, the correlation between severities of thought disorder and deficit of sensorimotor gating in schizophrenia patients is used as evidence that impairment in sensorimotor gating mechanisms contributes to these symptoms of schizophrenia.\textsuperscript{29} Given that the animals in this study were evaluated for the PPI of startle response at the age when cortical synaptic formation in rats achieves its maximum\textsuperscript{24,30} and that the cortex is involved in processing the PPI of startle,\textsuperscript{21} the findings of a reduced PPI suggest that exposure of rats to sevoflurane anesthesia during the early neonatal period may affect brain development.

Our current data support the previously reported finding of a role for sevoflurane-caused neuronal excitation\textsuperscript{4} and extend further to a possibility that a systemic action of the anesthetic may be involved in developmental neurotoxicity. The diagram in figure 5 is designed to illustrate hypothetical pathways that might mediate the side effects of neonatal anesthesia with sevoflurane that were observed in this study. The effects of bumetanide imply that the neuronal NKCC1-GABA_\text{\textsubscript{A}R} mechanisms may also play an important role in the abnormalities caused by sevoflurane. Bumetanide, which is the most specific inhibitor of neuronal NKCC1, has been used in numerous studies to investigate depolarizing excitatory actions of GABA in early postnatal brain.\textsuperscript{6,24} It previously was shown that long-term treatment of mice with bumetanide, from gestational day 15 through P7, resulted in disruption of the PPI of startle.\textsuperscript{24} Obviously, such different experimental conditions make it difficult to directly compare our results with those findings, but they support involvement of the NKCC1-GABA_\text{\textsubscript{A}R} mechanism in the regulation of sensorimotor gating function during brain development in rodents. Furthermore, our findings of disruption of the PPI of startle by sevoflurane, administered during the early postnatal period, are in line with reports that a single treatment of neonatal rats with positive GABA_\text{\textsubscript{A}R} receptor modulators, such as the barbiturate phenobarbital or the neurosteroid allopregnanolone, results in similar long-term impairment of the PPI of startle.\textsuperscript{31,32} A role for NKCC1-GABA_\text{\textsubscript{A}R} mechanisms in the side effects of sevoflurane is further indirectly supported by the finding that a single dose of bumetanide, without anesthesia, did not change the PPI of startle (fig. 1C). The inhibitory effect of bumetanide on neuronal activity should be greater in the presence of sevoflurane because of the sevoflurane-induced increase of Cl\textsuperscript{-} conductance through the GABA_\text{\textsubscript{A}R} channels.

The increase in serum concentrations of aldosterone caused by sevoflurane (fig. 1C) and the exacerbation of the sevoflurane-caused acute and long-term side effects by exogenous aldosterone (figs. 2 and 3) suggest that sevoflurane acts not only in the brain, but also at the peripheral sites (an increase in blood aldosterone concentrations), and neonates with disease states that are characterized by increased concentrations of aldosterone (e.g., premature birth, sepsis, inflammation, cardiovascular disorders)\textsuperscript{14,17–19} may be especially vulnerable to the harmful effects of sevoflurane. The exact molecular mechanism(s) whereby sevoflurane increases serum concentrations of aldosterone remains to be determined. The components of the GABA-ergic system, including GABA_\text{\textsubscript{A}R} proteins, were found in rat and human adrenal cortex cells.\textsuperscript{8,9} Furthermore, a functional role of these receptors in the adrenal gland is evident from the finding that both GABA and isoflurane increased the secretion of catecholamines in bovine adrenal cells by stimulation of GABA_\text{\textsubscript{A}R}-mediated depolarization.\textsuperscript{33} GABA still excited bovine chromaffin cells from 4–5-month-old animals,\textsuperscript{33} whereas depending on the strain of rats, the switch from excitatory to inhibitory action of GABA in rat cortical and hippocampal neurons occurs between postnatal days 8 and 13.\textsuperscript{34} An NKCC1-GABA_\text{\textsubscript{A}R}-mediated mechanism of sevoflurane-induced increase in serum concentrations of aldosterone is in disagreement with our findings that bumetanide increased serum concentrations of aldosterone beyond the increase already caused by sevoflurane (fig. 1D). However, the effect of bumetanide on the transmembrane gradient of Cl\textsuperscript{-} in the adrenal gland cells may be diminished because the expression of the K\textsuperscript{+}/Cl\textsuperscript{-} cotransporter, which promotes a developmental shift from depolarizing to inhibitory action of GABA, was not found in adrenal cells even in the juvenile animals.\textsuperscript{33} Furthermore, bumetanide has been shown to increase release of aldosterone in rats by stimulating renin secretion.\textsuperscript{35}
though aldosterone was shown to enhance NKCC1 activity in rat ventricular myocytes and vascular smooth muscle cells, a neuronal NKCC1-independent mechanism of action of aldosterone is supported by the finding that bumetanide failed to diminish and in some animals further enhanced seizure-like electroencephalogram patterns enhanced by exogenous aldosterone. Bumetanide, by increasing aldosterone release, may further contribute to NKCC1-independent excitatory effects of aldosterone. Poor permeability of the blood-brain barrier for aldosterone may explain the additive effect of exogenous aldosterone on the side effects of neonatal sevoflurane anesthesia, despite a remarkable increase in serum aldosterone concentrations caused by sevoflurane. Poor permeability of the blood-brain barrier for aldosterone may also be one of the reasons for the absence of an effect of exogenous aldosterone on concentrations of activated caspase-3 and the PPI of startle in nonanesthetized pups (fig. 3, B and C). General anesthesia may compromise the blood-brain barrier’s function and allow greater access for aldosterone to the brain. Failure of exogenous aldosterone to affect the electroencephalographic activity in older rat pups (fig. 2D), which may occur because of higher occupancy of mineralocorticoid receptors by glucocorticosteroids, supports the idea of developmental regulation of the action of aldosterone in the brain.

The therapeutic effects of oxytocin or its synthetic analog carbetocin also support sevoflurane-caused excitation as the mechanisms for its developmental side effects in rat pups. Oxytocin produces neuroprotective effects on the fetal brain by inhibiting NKCC1 activity and shifting GABAAR-mediated signaling from excitatory to inhibitory. In addition, oxytocin is known to produce a number of other effects opposite to those caused by aldosterone, such as a decrease of plasma aldosterone concentrations and antiinflammatory, anxiolytic, and antidepressant effects.

Altogether, our findings of excitatory effects of sevoflurane and aldosterone, as evident from the electroencephalogram patterns, and the alleviating effects of bumetanide suggest that the side effects of sevoflurane in neonatal rats result from depolarizing and/or excitatory actions of the anesthetic. Our results do not exclude that the sevoflurane-caused depolarization or excitation may also be caused via mechanisms other than enhancement of GABAAR activity. We further hypothesize that depolarization or excitation is mediated in involvement of the developmental side effects produced by anesthetics with molecular mechanisms of action similar to those of sevoflurane.

The following reported findings support this hypothesis. In rodents, propofol- and volatile anesthetic-caused neurodegeneration, impairment of neuronal progenitor cell proliferation, and cognition diminish when GABAAR-mediated signaling switches from depolarizing to inhibitory. This is in line with the functional role of early depolarizing GABA, which is known to inhibit proliferation and migration of neuronal precursors. Depolarizing GABA also promotes neurite outgrowth and synaptogenesis. The last notion seems to contradict the hypothesis of excitatory mechanisms of developmental effects of propofol because propofol decreased spine density in the medial prefrontal cortex of the rats through postnatal day 10 but caused opposite effects in rats starting at postnatal day 15. However, because propofol caused opposite effects in younger and older rats, it is reasonable to suggest that GABAAR-mediated excitatory and inhibitory actions of propofol were involved. Finally, an excitatory hypothesis of neonatal anesthesia toxicity is supported indirectly by findings in neonatal rhesus macaques, in which isoflurane caused neurodegeneration predominantly in the cerebral cortex, whereas ketamine caused it in both cortical and subcortical structures. The expression patterns of NKCC1 and KCC2 from rodents to humans suggests that shortly after birth GABA is excitatory in neocortical neurons but inhibitory in subcortical structures. It is plausible that the greater neurotoxic effect of ketamine during early stages of brain development may be, at least in part, attributable to different subunit composition of N-methyl-D-aspartate receptors at different stages of brain development. Prenatal and early postnatal receptors contain exclusively NR2B subunits, whereas increased incorporation of NR2A subunits occurs during the second postnatal week. NR2B knockout animals die on P0, but NR2A knockout mice are normal. Ketamine-caused increases in cortical neuronal activity, which may be even greater in neonatal cortex because of depolarizing GABA, also potentially may contribute to neonatal toxicity. In summary, our results suggest that developmental effects of neonatal anesthesia with sevoflurane may involve both central and peripheral actions of the anesthetic and subsequent increases in neuronal activity. Subjects with increased concentrations of aldosterone may be more vulnerable, whereas intracerebral oxytocin receptor agonists may be neuroprotective.

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