

# Bumetanide Alleviates Epileptogenic and Neurotoxic Effects of Sevoflurane in Neonatal Rat Brain

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## ABSTRACT

**Background:** We tested the hypothesis that in newborn rats, sevoflurane may cause seizures, neurotoxicity, and impairment in synaptic plasticity—effects that may be diminished by the  $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$  cotransporter 1 inhibitor, bumetanide.

**Methods:** Electroencephalography, activated caspase-3, and hippocampal long-term potentiation were measured in rats exposed to 2.1% sevoflurane for 0.5–6 h at postnatal days 4–17 (P4–P17).

**Results:** Arterial blood gas samples drawn at a sevoflurane concentration of 2.1% showed no evidence of either hypoxia or hypoventilation in spontaneously breathing rats. Higher doses of sevoflurane (e.g., 2.9%) caused respiratory depression. During anesthesia maintenance, the electroencephalography exhibited distinctive episodes of epileptic seizures in 40% of P4–P8 rats. Such seizure-like activity was not detected during anesthesia maintenance in P10–P17 rats. Emergence from 3 h of anesthesia with sevoflurane resulted in tonic/clonic seizures in some P10–P17 rats but not in P4–P8 rats. Bumetanide (5  $\mu\text{mol/kg}$ , intraperitoneally) significantly decreased seizures in P4–P9 rats but did not affect the emergence seizures in P10–P17 rats. Anesthesia of P4 rats with sevoflurane for 6 h caused a significant increase in activated caspase-3 and impairment of long-term potentiation induction measured at 1 and 14–17 days after exposure to sevoflurane, respectively. Pretreatment of P4 rats with bumetanide nearly abolished the increase in activated caspase-3 but did not alleviate impairment of long-term potentiation.

**Conclusion:** These results support the possibility that excitatory output of sevoflurane-potentiated  $\gamma$ -aminobutyric acid type A/glycine systems may contribute to epileptogenic and neurotoxic effects in early postnatal rats.

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## What We Already Know about This Topic

- ❖ Anesthetics can cause seizures and programmed cell death (apoptosis) in neonatal animals
- ❖  $\gamma$ -Aminobutyric acid (GABA) can stimulate rather than inhibit immature neurons, because of a developmental change in chloride gradients

## What This Article Tells Us That Is New

- ❖ In neonatal rats, sevoflurane caused seizures and apoptosis that were blocked by administration of a blocker of chloride uptake

**H**EMODYNAMIC stability, lack of respiratory irritation, and short-lasting action make sevoflurane one of the most widely used general anesthetics, particularly in pediatric anesthesia. Unfortunately, sevoflurane, similar to some other general anesthetics, has been reported to cause epileptiform electroencephalographic activity and seizure-like movements.<sup>1–8</sup> The hyperexcitatory events in neonates and infants are of tremendous concern because they may potentially result in delayed neurologic and cognitive defects.<sup>9</sup> Recent data indicate that a single episode of neonatal seizures is sufficient to cause permanent alterations in memory long after the initial seizure episode in a neonatal rat model.<sup>10</sup>

The major known effects of sevoflurane in the brain include activation of various two-pore-domain  $\text{K}^+$  channels, depression of glutamate release, inhibition of nicotinic acetylcholine receptors, and enhancement of many types of  $\gamma$ -aminobutyric acid ( $\text{GABA}_A$ ) and strychnine-sensitive glycine receptors.<sup>11–14</sup> All these effects, at least in their classic understanding, lead to inhibition and induction of the anesthetic state but by themselves should not cause excessive excitatory phenomena as may be observed during and imme-

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◆ This article is accompanied by an Editorial View. Please see: Stratmann G: Aneseizure: Is the neonatal brain asleep? ANESTHESIOLOGY 2010; 112:527–9.

diately after anesthesia with sevoflurane. On the other hand, the changes in the transmembrane gradient of  $\text{Cl}^-$ , which is the charge carrier through  $\text{GABA}_A$  and strychnine-sensitive glycine receptor channels,<sup>15,16</sup> can reverse the output of activation of these receptors from inhibitory to excitatory and, therefore, may play an important role in the hyperexcitation phenomena associated with sevoflurane anesthesia. The observations that all general anesthetics that have a  $\text{GABA}_A$  component of action may cause episodes of hyperexcitation indirectly support this possibility.<sup>1-8</sup>

In comparison with mature neurons, neurons at early stages of postnatal development have significantly increased intracellular concentrations of  $\text{Cl}^-$ ,  $[\text{Cl}^-]_i$ , and as a result, the equilibrium potential for  $\text{Cl}^-$ ,  $E_{\text{Cl}}$ , is more positive than the resting membrane potential. Therefore, during early development, activation of  $\text{GABA}_A$  receptors results in  $\text{Cl}^-$  efflux and neuronal depolarization.<sup>17-19</sup> In fact,  $\text{GABA}_A$  receptor activation is recognized as a major source of excitation at early stages of brain development.<sup>20</sup>

Uptake of  $\text{Cl}^-$  by the  $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$  cotransporter isoform 1 (NKCC1) has been shown to provide the driving force for depolarizing  $\text{GABA}_A$  receptor-mediated responses in immature neurons.<sup>16-20</sup> The peak expression of NKCC1 in rodents is around postnatal days 5-7 (P5-P7).<sup>19</sup> Consistent with the specific role of NKCC1 in the depolarizing effects of GABA, GABA-mediated depolarization in immature neurons was shown to be blocked by bumetanide, a specific inhibitor of NKCC1 at low doses.<sup>19,21</sup> Bumetanide also inhibits cortical seizure activity in neonatal rats *in vitro* and *in vivo*. The ontogenetic shift to a hyperpolarizing action of GABA is caused by a concomitant developmental down-regulation of NKCC1 and an up-regulation of the  $\text{K}^+ - \text{Cl}^-$  cotransporter isoform 2 (KCC2).<sup>22,23</sup> KCC2 expression is not detectable in rat neurons after birth, and its expression during the second postnatal week is associated with a progressive negative shift in the reversal potential of  $\text{GABA}_A$  receptor-mediated responses and a switch in the action of GABA from excitatory to inhibitory.<sup>21,24</sup>

We present results that support the theory that excitatory output of sevoflurane-potentiated  $\text{GABA}_A$ /glycine systems may contribute to the epileptogenic and neurotoxic effects of sevoflurane in early postnatal rats.

## Materials and Methods

### Animals

All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee (Gainesville, FL). In addition, the principles governing the care and treatment of animals, as stated in the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (publication No. 85-323, revised 1996), were followed at all times during this study. Sprague-Dawley rats were studied. To control for litter variability, we used several pups for each treatment condition from each litter. At the beginning of each experiment, the pups were determined to be well nourished, judged by their stomachs being full of milk (detectable through the transparent abdominal wall).

### Anesthesia and Electroencephalogram Recording

To determine the effects of sevoflurane on cortical activity, rat pups ranging from P4-P17 were instrumented for electroencephalogram recording. Half of the  $\leq$  P5 rats were anesthetized by cold immersion (to avoid additional exposure to anesthetic), and the other half and older rats were anesthetized by inhalation anesthesia with isoflurane (1.8-2.5%). Using bregma as needed for a reference, four holes (0.5 mm) were burred (Microtorque II; WPI, Sarasota, FL) in the skull at bilateral frontal and occipital regions for implantation of four electrodes of the headmounts of the electroencephalogram/electromyogram system (Pinnacle Technology, Lawrence, KS; fig. 1A). The entire instrumentation for electroencephalogram recording took no more than 20 min. Electroencephalogram recording started immediately after completion of the implant surgery and ended 30 min

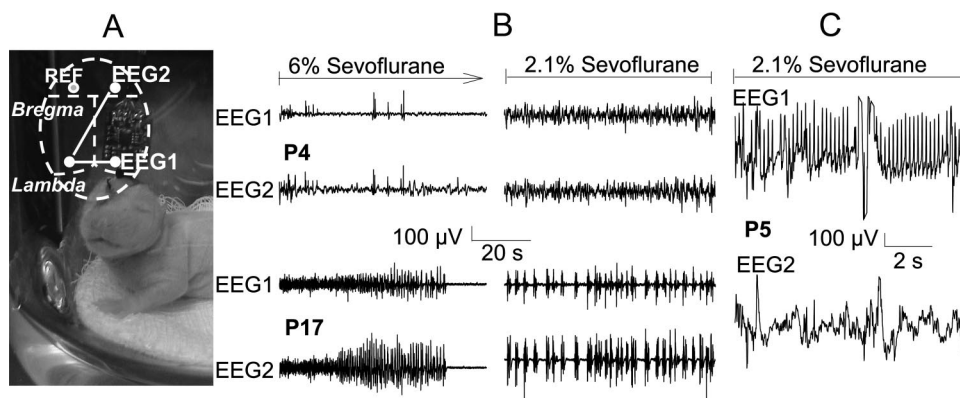


Fig. 1. Electroencephalographic (EEG) activity in rat pups during sevoflurane anesthesia. (A) An illustration of the EEG electrode placement for continuous EEG recordings from bilateral occipital (EEG1) and right frontal (EEG2) regions using an EEG/electromyogram system (Pinnacle Technology, Lawrence, KS). (B) Examples of EEG recordings in postnatal days 4 and 17 (P4 and P17) rats during start of anesthesia induction with 6% sevoflurane and during anesthesia maintenance with 2.1% sevoflurane. (C) An example of EEG seizures recorded from a 5-day-old male rat during anesthesia with 2.1% of sevoflurane (see Results for details). REF = reference electrode.

after termination of anesthesia with sevoflurane. The 60-min anesthesia-free interval after the implant surgery before exposure to sevoflurane was allowed to record a baseline electroencephalogram. Sevoflurane (Fushimi-Machi, Osaka, Japan) anesthesia was induced with 6% sevoflurane and 1.5 l/min oxygen over 3 min and maintained with 2.1% sevoflurane and 1.5 l/min oxygen over 30–360 min in a thermostated chamber. Backdraft through wall vacuum was used to scavenge waste gases. 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 that sampled from the interior of the animal chamber. To ensure adequate oxygenation and respiration, some of the rat pups were anesthetized with the same sevoflurane protocol as rats in the study group, and arterial blood gas and glucose determinations were performed by cardiac puncture at the conclusion of the anesthetic. Arterial blood gases and glucose were measured using a portable clinical analyzer (i-STAT; Abbott Laboratories Inc., East Windsor, NJ). Continuous electroencephalogram recordings from bilateral occipital and right frontal regions in rat pups were performed using an electroencephalogram/electromyogram system (Pinnacle Technology). Acquisition of the electroencephalogram was performed using the Sirenia software (Pinnacle Technology). Sampling interval per signal was 200  $\mu$ s (5 kHz). Sirenia Score (Pinnacle Technology) and Clampfit 9.2 (Axon Instruments, Union City, CA) programs were used for the electroencephalogram data analysis. Data were filtered off-line using a bandpass Bessel (8-pole) 0.04–56-Hz filter. Power spectrum analysis, revealing the power levels of different frequency components in the signal, was performed after applying a Hamming window function. Power was calculated in 1- to 2-min time windows by integrating the root mean square value of the signal in frequency bands. Electroencephalographic seizures were defined as electroencephalogram patterns of high-amplitude rhythmic activity with evolution in frequency or amplitude that were at least three times higher than the baseline activities, lasted for at least 3–10 s, and abruptly reverted to baseline.

### **In Vitro Electrophysiological Recordings**

Sprague-Dawley rats (P4–P6) were decapitated (P18–P22 rats were deeply anesthetized with isoflurane before decapitation), and the brains were dissected in cold (0°–4°C) oxygenated (95% O<sub>2</sub>–5% CO<sub>2</sub>) artificial cerebral spinal fluid containing 120 mM NaCl, 3 mM KCl, 25 mM NaHCO<sub>3</sub>, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, 2 mM MgSO<sub>4</sub>, and 20 mM glucose (300 mOsm). Hippocampal coronal brain slices (400  $\mu$ m) were cut with a vibrating-blade microtome Leica VT1000S (Leica Biosystems GmbH, Nussloch, Germany). The slices were maintained at room temperature for  $\geq$  1 h in an interface holding chamber filled with humid 95% O<sub>2</sub>–5% CO<sub>2</sub> before transfer to a submersion experimental chamber superfused at 2 ml/min with artificial cerebral spinal fluid at 32°–33°C.

Schaffer collaterals were stimulated at 0.05 Hz (100  $\mu$ s, 20–40  $\mu$ A) with a concentric bipolar stimulating electrode, and field excitatory postsynaptic potentials (fEPSPs) were recorded in the CA1 stratum radiatum using glass electrodes filled with 2 M NaCl (3–6 M $\Omega$  resistance). Healthy slices were identified by the presence of a single population spike at a wide range of stimulus intensities and by observing stable field potentials more than 1 mV requiring a similar amount of current for a period of 5 min before recording the experimental baseline. Slices not meeting these criteria were excluded. Baseline fEPSPs were set to 40% of maximum response. A stable baseline was recorded for at least 15 min. Long-term potentiation (LTP) was induced using a high-frequency stimulation (HFS) protocol (two 1-strains at 100 Hz, separated by 20 s). Data were collected using an Axopatch 1D amplifier (Axon Instruments), filtered at 2 kHz and digitized at 5 kHz using a Digidata 1200 and Clampex 8 software (Axon Instruments). The slope of the fEPSP was measured after the end of the fiber volley in a 1-ms window. LTP was calculated as follows: LTP (%) = 100  $\times$  (mean slope of 10 fEPSPs 30 min after HFS/mean slope of 10 fEPSPs before HFS). Paired pulse ratio of the fEPSP slope was obtained by stimulating two sequential fEPSPs per trace, 80 ms apart (fEPSP1 and fEPSP2), and calculated using the average of 10 traces with the following formula: paired pulse ratio = fEPSP2/fEPSP1. For experiments studying the effects of acute exposure to sevoflurane, the anesthetic was bubbled into the bath solution using a calibrated Penlon-Sigma vaporizer (Penlon, Abingdon, United Kingdom).

### **Determination of Activated Cleaved Caspase-3 Using Western Blot**

Eighteen hours after exposure to sevoflurane, rat pups were deeply anesthetized with isoflurane and perfused with phosphate-buffered saline through the left cardiac ventricle. The brains were removed from the skull, put into liquid nitrogen, and then stored at –80°C until further use. On the day of analysis, the brain tissue was allowed to equilibrate to a temperature of +4°C. The tissue samples were then homogenized in 10% (w/v) Radio-Immunoprecipitation Assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing complete protease inhibitors (Sigma, St. Louis, MO) and centrifuged at 16,000g for 20 min. The supernatants were collected and quantified for protein concentration by bicinchoninic acid assay method (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Prepared protein samples (375  $\mu$ g/well) were separated on 8–16% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). The membranes were blocked with 5% nonfat milk in 0.01 M phosphate-buffered saline with 0.1% Tween-20 (pH 7.4) at room temperature for 1 h. Then, the membrane was incubated at 4°C overnight with cleaved caspase-3 antibodies (Cell Signaling, Danvers, MA; 1:500, diluted with 5% nonfat milk in 0.01 M phosphate-buffered saline with 0.1% Tween-20). After three washes in 0.01 M phosphate-buffered

saline with 0.1% Tween-20, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted to 1:2000 in phosphate-buffered saline with 0.1% Tween-20 for 1 h at room temperature. After three washes in 0.01 M phosphate-buffered saline with 0.1% Tween-20 again, the blots were detected with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Rockford, IL). To prove equal loading, the blots were analyzed for  $\alpha$ -tubulin expression using an anti- $\alpha$ -tubulin antibody (Santa Cruz Biotechnology, Inc.). Exposure time was 10 min for caspase-3 and 20 s for  $\alpha$ -tubulin. The bands were semiquantified with J-image software (National Institutes of Health, Bethesda, MD). Western blot analysis for tissue samples from each animal was performed in triplicate and reported as an average.

### Statistical Analysis

Values are reported as mean  $\pm$  SEM. SigmaStat 3.11 software (Systat Software, Inc., 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 over time in multiple groups were analyzed using repeated-measures ANOVA followed by Holm-Sidak tests. Differences in proportions among groups were analyzed with a *z* test. A *P* value less than 0.05 was considered significant.

## Results

### Seizure-like Electroencephalographic Activity in P4–P17 Rats during Sevoflurane Anesthesia: Effects of Bumetanide

Arterial blood gas samples drawn at sevoflurane concentration of 2.1% showed no evidence of either hypoxia or hypoventilation ( $PO_2$ :  $429 \pm 8$ ;  $PCO_2$ :  $46 \pm 4$ ; glucose:  $118 \pm 8$ ); on the other hand, sevoflurane at 2.9% caused respiratory depression in spontaneously breathing rats. Because hypercarbia itself may increase seizure susceptibility<sup>25,26</sup> and thus interfere with data interpretation of epileptogenic effects of sevoflurane, the concentration of 2.1% sevoflurane was used as the maintenance dose throughout the whole project, whereas 6% sevoflurane for 3 min was used for anesthesia induction. Based on data from the study by Orliaguet *et al.*,<sup>27</sup> minimum alveolar concentration for sevoflurane in 9- and 2-day-old rats is 3.74 and 3.28%, respectively. Therefore, 2.1% sevoflurane as used in this study lies near the 0.6 minimum alveolar concentration.

First, seizure-like electroencephalographic activity was evaluated during 3 min of anesthesia induction with 6% sevoflurane and 30 min of anesthesia maintenance with 2.1% sevoflurane in 39 rat pups that ranged in age from P4 to P17. Start of induction of anesthesia with 6% sevoflurane was associated with a brief increase in electroencephalogram frequency followed by a gradual increase in amplitude and decrease in frequency that was followed by an abrupt and almost complete loss of electrical activity in older pups (fig. 1B). There was no such distinct transitional period during

sevoflurane induction in younger rats. A decrease in sevoflurane concentration from an induction dose of 6% to a maintenance dose of 2.1% resulted in an increase in amplitude and frequency of the electroencephalogram in younger animals and caused burst suppression-like activity in some older rat pups (fig. 1B). Only one (P5) of the 39 rats studied (P4–P17) exhibited a 58-s episode of seizure-like electroencephalographic activity during 3 min of anesthesia induction with 6% sevoflurane. During the first 30 min of anesthesia maintenance with 2.1% sevoflurane, eight of the 20 P4–P8 rats or 40% of rats of this age category exhibited electroencephalographic seizures that lasted from 10 s in one animal (P6) to 20.8 min in another (P5), with a mean duration of  $86 \pm 62$  s ( $n = 20$ ). This seizure-like activity could be detected by recording both electroencephalogram 1 and 2; most frequently, it was present in electroencephalogram 2 only, although some animals exhibited seizure-like activity in electroencephalogram 1 but not in electroencephalogram 2 (fig. 1C). Within this age category, younger rats exhibited episodes of seizure-like electroencephalographic activity more frequently than their older counterparts. Thus, only one of the six P7 rats had seizures, whereas seizures were observed in six of the 12 P4–P6 rats. This trend in frequency of episodes of seizure-like activity in electroencephalogram continued in P10–P17 rats. These rat pups ( $n = 19$ ) did not exhibit any seizure-like electroencephalographic activity either during 3 min of 6% sevoflurane or during 30 min of 2.1% sevoflurane.

Next, we tested the possibility that increased concentrations of intracellular  $Cl^-$ , accumulated by NKCC1, are responsible for excitatory effects of sevoflurane in rats at earlier stages of brain development. The sulfamyl loop diuretic bumetanide at low concentrations is currently the only selective NKCC1 transporter inhibitor. The effect of bumetanide on frequency and duration of episodes of seizure-like activity during sevoflurane anesthesia was studied in P4–P9 rats. The P4–P9 rats were randomly distributed between two groups with similar number of animals of same age in both groups. The animals in group 1 received bumetanide (5  $\mu$ mol/kg, intraperitoneally) 15 min before anesthesia induction with 6% sevoflurane for 3 min followed by anesthesia maintenance with 2.1% sevoflurane for another 60 min. Rats in group 2 received the same volume of saline. Seizures were observed in one of the 11 animals that received bumetanide and in eight of the 13 rats that received saline. Furthermore, the duration of seizure episodes in the bumetanide-treated rats was significantly decreased (fig. 2).

In some older rat pups ( $> P10$ ), the emergence from 3 h of anesthesia with 2.1% sevoflurane resulted in more intensive seizures than those that were observed in P4–P8 rats during sevoflurane anesthesia. In contrast to seizures during anesthesia in younger rat pups, which were not readily associated with muscle movement, electroencephalographic seizures during emergence from anesthesia in older rat pups were accompanied by clonic/tonic muscle movements. Usually, these emergence seizures occurred in several episodes

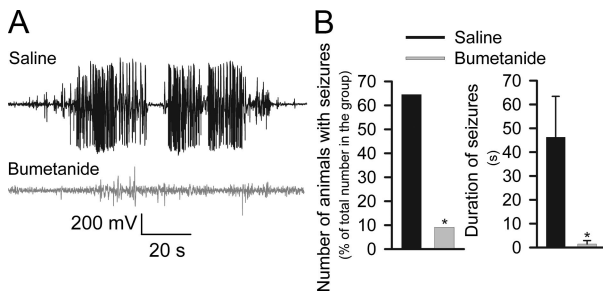


Fig. 2. Bumetanide, the  $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$  cotransporter 1 (NKCC1) inhibitor, depressed seizures during sevoflurane anesthesia in postnatal days 4–9 (P4–P9) rats. (A) Examples of electroencephalogram recordings during 2.1% sevoflurane anesthesia in saline-pretreated rat (P4, top) and bumetanide-pretreated rat (P4, bottom). Two groups of animals (P4–P9) received either bumetanide ( $5 \mu\text{mol/kg}$ , intraperitoneally) or an equal volume of saline 15 min before anesthesia with 6 and 2.1% sevoflurane that lasted for 3 and 60 min, respectively. Only one rat from the bumetanide-pretreated group exhibited 16 s long seizures during 60 min 2.1% sevoflurane. (B) Summary data for the experiment presented in A. Bumetanide decreased number of animals that had seizures during anesthesia and duration of seizures. \*  $P < 0.05$  vs. saline plus sevoflurane ( $n = 11$  and  $13$  in the bumetanide- and saline-pretreated groups, respectively).

starting between approximately 1 and 15 min after termination of sevoflurane administration (fig. 3). These seizures lasted from 50 s in one animal (P13) to 840 s in another one (P14), with a mean duration of  $486 \pm 133$  s ( $n = 6$ ). Such emergence seizures were not prevented by pretreatment with bumetanide administered 30 min before termination of anesthesia. To test the possibility that the observed emergence seizures are caused by low, subanesthetic doses of sevoflurane that may occur during terminal elimination of sevoflurane after the end of an anesthetic, we studied the effects of 0.1, 0.2, and 0.5% sevoflurane applied for 60 min in P10–P17 rats. These low doses of sevoflurane neither caused seizures nor any obvious increase in electroencephalographic activity in these animals.

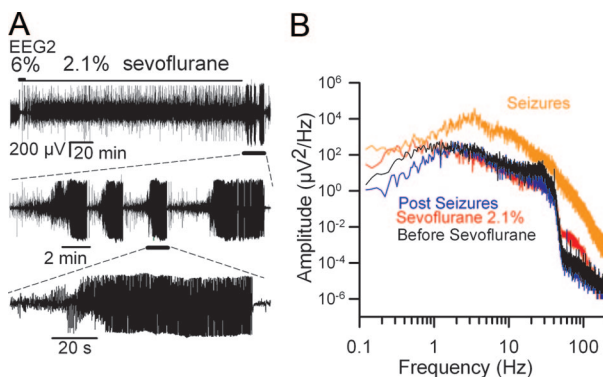


Fig. 3. Electroencephalographic (EEG) seizures during emergence from 3-h long anesthesia with 2.1% sevoflurane. (A) An example of EEG2 recording from a 14-day-old male rat. (dotted lines) The episode of the EEG that corresponds to the fragments of EEG recording is shown above. (B) Power spectrums for each stage of the experiment.

### Bumetanide Diminishes Activation of Caspase-3 in the Brain of Neonatal Rat Pups Exposed to Sevoflurane

In this study, we assessed whether sevoflurane anesthesia in neonatal rat pups may cause neurotoxicity and whether this toxicity can be decreased by bumetanide. Neonatal rats (P4) were exposed to sevoflurane as described above: 6% sevoflurane for 3 min for induction and 2.1% sevoflurane for 360 min for anesthesia maintenance. One half of these rats received bumetanide ( $5 \mu\text{mol/kg}$ , intraperitoneally) 15 min before anesthesia with sevoflurane; the remainder received the same volume of saline. Rats in the control group were not exposed to sevoflurane. Again, absence of hypoxia and hypoventilation was assessed in a separate group of rat pups, each anesthetized for 360 min and subjected to arterial blood gas sampling by cardiac puncture at the conclusion of the anesthetic. After emergence from anesthesia, rats were tagged and returned to their dam. Eighteen hours after emerging from anesthesia, animals were killed, and apoptotic changes in the brain were determined by evaluating activated caspase-3 using a Western blot technique. Caspase activity was significantly increased in brain tissue of the sevoflurane-anesthetized rats that received saline before exposure to sevoflurane. In contrast, animals that received bumetanide before exposure to sevoflurane had activated cleaved caspase-3 signals comparable with control rats, animals that were not exposed to sevoflurane anesthesia (fig. 4).

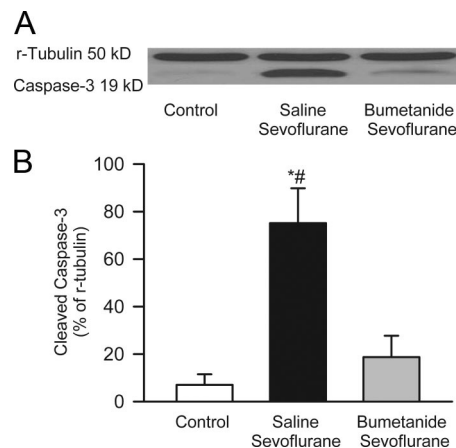


Fig. 4. Sevoflurane anesthesia of neonatal rats increases caspase-3 activation, an effect that is diminished by pretreatment with bumetanide. Postnatal day 4 (P4) rats before exposure to 6 h of sevoflurane (2.1%) anesthesia were pretreated either with  $5 \mu\text{mol/kg}$  bumetanide (sevoflurane + bumetanide,  $n = 4$ ) or with an equal volume of saline (saline + sevoflurane,  $n = 4$ ). Rats in the control group (control,  $n = 5$ ) did not undergo anesthesia on P4. Brains were isolated on P5 for cleaved caspase-3 evaluation. (A) Representative Western blot images of cleaved caspase-3 in the brain of all three groups of rats to illustrate band intensities. (B) Histogram showing results of the densitometric analysis of Western blot images of cleaved caspase-3. Densities of r-tubulin blots from the same tissue sample were taken as 100%. \*#  $P < 0.05$  vs. control and sevoflurane plus bumetanide, respectively.

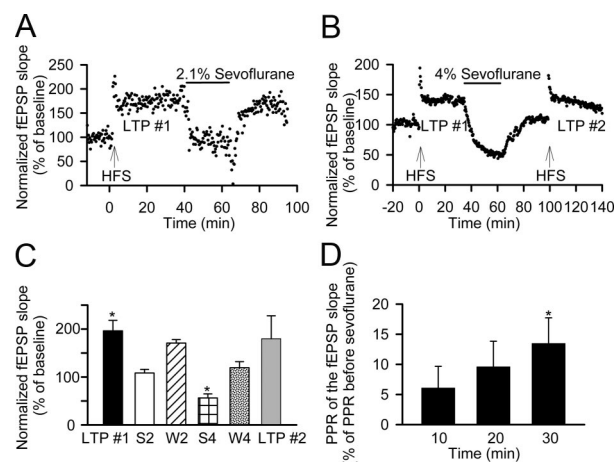


Fig. 5. Acute effects of sevoflurane on hippocampal long-term potentiation (LTP). (A) A representative experiment showing the time course of changes of field excitatory postsynaptic potential (fEPSPs) slope caused by high-frequency stimulation (HFS) and by subsequent application of sevoflurane. Thirty minutes after HFS, 2.1% sevoflurane was added to the bath for 20 min (*horizontal bar*). (B) A representative experiment with 4% sevoflurane. After washout of sevoflurane, the fEPSP slope remained near baseline. At 100 min, a second HFS pulse resulted in LTP again. (C) Group data: S2, sevoflurane at 2.1%; W2, after 30 min washout of 2.1% sevoflurane; S4, sevoflurane at 4%; W4, after 30 min washout 4% sevoflurane; LTP #2, in two of four experiments using 4% sevoflurane, LTP was evoked again. \*  $P < 0.01$  vs. baseline (pre-HFS). (D) Group data of percent change in paired pulse ratio (PPR) of the fEPSP slope at 10, 20, and 30 min after 4% sevoflurane exposure. \*  $P < 0.01$  vs. PPR before sevoflurane exposure.

### Neonatal Exposure to Sevoflurane Transiently Reduces LTP during Development

First, the acute effects of sevoflurane on LTP were studied in hippocampal slices prepared from the brains of P4–P6 rats (fig. 5). LTP was induced by HFS (see Materials and Methods section for details). Thirty minutes after HFS, LTP was measured by the slope of the fEPSP and found to be increased to  $196.3 \pm 21.8\%$  ( $n = 6$ ,  $P < 0.01$ ) compared with baseline. After LTP had been established, slices were exposed to sevoflurane. After 20 min of exposure to 2.1% sevoflurane, fEPSPs decreased to  $108 \pm 8\%$  of control (pre-HFS) and reverted to  $171 \pm 7\%$  ( $n = 2$ ) after washout. Sevoflurane at 4% decreased fEPSPs to 56% of control (pre-HFS). Interestingly, after sevoflurane washout, the fEPSP slope returned to near baseline levels of  $119 \pm 13\%$  ( $n = 4$ ). In two of those slices, HFS was performed a second time and LTP was re-established to  $180 \pm 49\%$  after 30 min. During wash-in of 4% sevoflurane, the paired-pulse ratio of the fEPSP slope increased compared with paired pulse ratio during LTP 1 by  $6 \pm 4\%$  after 10 min,  $10 \pm 4\%$  after 20 min, and became significant to  $14 \pm 4\%$  after 30 min ( $n = 6$ ,  $P < 0.01$ ), indicating the possibility of a presynaptic action of 4% sevoflurane.

Next, we assessed whether neonatal exposure to sevoflurane has delayed effects on induction of LTP during development and whether this effect of sevoflurane can be reversed by pretreatment of neonatal rats with bumetanide. To test

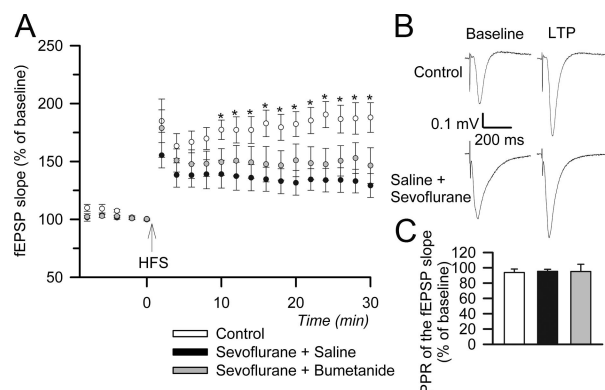


Fig. 6. Long-term effect of sevoflurane anesthesia in neonatal rats on hippocampal long-term potentiation (LTP). Postnatal day 4 rats were administered bumetanide ( $5 \mu\text{mol/kg}$ , intraperitoneally) or equal volumes of saline 15 min before exposure to 2.1% sevoflurane for 6 h. Control rats were not exposed to sevoflurane. Hippocampal slices were isolated from the brains of these rats at postnatal days 18–22 (P18–P22), and field excitatory postsynaptic potentials (fEPSPs) were recorded. (A) The time course of changes in the slope of fEPSPs caused high-frequency stimulation (HFS) in hippocampal slices from all three treatment groups (control,  $n = 6$ ; saline plus sevoflurane,  $n = 11$ ; bumetanide plus sevoflurane,  $n = 4$ ). Each symbol represents averaged slope of fEPSPs recorded during the 2-min period. (B) Representative fEPSP traces recorded before and 30 min after HFS show reduced LTP in sevoflurane exposed traces. (C) Paired pulse ratio (PPR) of the fEPSP slope after HFS in hippocampal slices from all three treatment groups.

this possibility, rat pups were exposed to 2.1% sevoflurane at P4, for 6 h as described above (see the preceding section). Fifteen minutes before anesthesia with sevoflurane, the rats received either bumetanide ( $5 \mu\text{mol/kg}$ , intraperitoneally) or an equal volume of saline. Once these rats reached juvenile age (P18–P22), they were killed and LTP was measured in hippocampal slices. Compared with nonexposed littermates, animals exposed to sevoflurane that received an injection of saline as a pretreatment had reduced LTP. The fEPSP slopes for sevoflurane-anesthetized and nonanesthetized animals were  $129 \pm 10\%$  and  $188 \pm 13\%$ , respectively ( $n = 11$  and  $n = 6$ ,  $P < 0.01$ , fig. 6). Although LTP in hippocampal slices from the sevoflurane-exposed animals pretreated with bumetanide was greater than that in the saline pretreated rats, it was not big enough to achieve statistical significance ( $146 \pm 15\%$ ,  $n = 4$ ). Interestingly, in contrast to the acute action of sevoflurane on LTP induction, the paired pulse ratio of the fEPSP slope was similar in hippocampal slices from control animals ( $94 \pm 5\%$ ,  $n = 6$ ) and those that were exposed to sevoflurane ( $96 \pm 3\%$ ,  $n = 11$ ) or sevoflurane plus bumetanide ( $95 \pm 10\%$ ).

### Discussion

The main finding of this study is that general anesthesia with sevoflurane may cause epileptic seizures and neurotoxicity in neonatal rats and that these effects could be diminished by the NKCC1 transporter inhibitor bumetanide. Bumetanide did not affect emergence seizures in older rat pups, indicating different underlying mechanisms in these two types of epi-

leptic seizures associated with sevoflurane anesthesia. Furthermore, an impairment of hippocampal long-term potentiation could be detected weeks after sevoflurane anesthesia in neonatal rats, an effect that was not significantly ameliorated by pretreatment with bumetanide.

Seizure-like phenomena related to anesthesia have been reported in patients of all ages from neonates through those in their eighties, although more frequently during early periods of life. Many of these seizures do not have a typical clinical phenotype and would therefore only be detected by electroencephalographic monitoring, which is not routinely performed. Therefore, the true incidence of seizures caused by anesthetics particularly in the youngest patients is not known but may be quite high even in the absence of central nervous system diseases or severe systemic illness.

Although a wide range of cellular mechanisms mediate the anesthetic action of sevoflurane, GABA<sub>A</sub>/glycine receptor-mediated Cl<sup>-</sup> currents seem to be a plausible mechanism to contribute to the excitatory effect of sevoflurane at early stages of brain development. The concentrations of bumetanide used in this study selectively inhibit NKCC1,<sup>19</sup> indicating that inhibition of Cl<sup>-</sup> accumulation is a probable mechanism by which bumetanide exerts its inhibition of the epileptogenic and neurotoxic effects of sevoflurane. Conversely, these data are consistent with the possibility that sevoflurane causes its epileptogenic and neurotoxic effects in the developing brain, at least in part, by potentiating GABA<sub>A</sub>/glycine receptor-mediated depolarizing Cl<sup>-</sup> currents. On the other hand, the well-known renal diuretic effect of bumetanide, although not specifically studied, is unlikely to explain our experimental findings. We did not observe an obvious increase in urine production in these rat pups after bumetanide administration. This may be, at least in part, because such an observation is difficult given the small body size of the rat pups or possibly because of the immature renal function at this age. In a human neonate, bumetanide exerted antiepileptic effects during 2 h of observation after a single dose with only slightly increased urine output.<sup>28</sup> If a nonspecific electrolyte-mediated effect on seizure threshold existed, then bumetanide should have also decreased emergence seizures in older rat pups, an effect not observed. The blood gas analyses performed in the control animals lend further support to the contention that the observed adverse effects are specific effects of the anesthetic and not related to perturbations caused by the experimental manipulations. Consistent with these effects of bumetanide are literature data that bumetanide decreased GABA-mediated depolarization in immature neurons and cortical seizure activity in neonatal rats both *in vitro* and *in vivo*.<sup>19,21</sup> NKCC1-mediated actions of bumetanide are also supported by the findings that NKCC1-mediated effects are not observed in the presence of antagonists of the GABA<sub>A</sub> receptors and that bumetanide did not affect epileptiform activity in brain slices from NKCC1<sup>-/-</sup> mice.<sup>19</sup> Our assumption that a single dose of bumetanide achieves a sufficient effect in the central nervous system is based on literature data, on its lipid or water solubility, and on the slow elim-

ination of bumetanide in neonatal patients. Pharmacokinetic studies of bumetanide have not been performed in neonatal rats; however, in preterm and full-term human neonates, bumetanide is reported to have a half-life of approximately 6 h, with a range up to 15 h.<sup>29</sup>

Experimental data on neuronal death in neonatal rodents caused by other anesthetics with a GABA<sub>A</sub>/glycine-ergic component of action also indirectly support a role of excitatory mechanisms in these effects. Thus, 6-h long sedation with isoflurane caused significant neurodegeneration in P7 rat pups.<sup>30</sup> Another study reported that P7 rats treated for 6 h with isoflurane exhibited significant and dose-dependent neurodegeneration, whereas P10 and P14 rats showed no significant increase in apoptotic neurodegeneration.<sup>31</sup> The P5–P7 period coincides with the peak expression of NKCC1 and peak [Cl<sup>-</sup>]<sub>i</sub>.<sup>19</sup> Therefore, an increase in neuronal activity at this developmental stage in the presence of isoflurane, whose mechanisms of action include activation of GABA<sub>A</sub>/glycine receptors, is plausible. In further support of this possibility are the findings reported by Nunez *et al.*<sup>32</sup> that GABA activation in newborn rats caused excessive calcium influx and cell death. In addition, a recent *in vitro* study indicates that propofol increased intracellular Ca<sup>2+</sup> concentration and neurotoxicity in cultured hippocampal neurons *in vitro* at day 4 but not at day 8.<sup>33</sup> Sevoflurane has also been shown to cause apoptosis and learning deficits and abnormal social behaviors in mice exposed to sevoflurane at P6–P7.<sup>34–36</sup>

Dzhala *et al.*<sup>19</sup> demonstrated that similar to rat pups, humans show high neuronal expression of NKCC1 and low expression of KCC2 before the end of the first year of life. This observation raises the possibility that our findings are applicable to human preemies and neonates. Interestingly, the developmental shift in the expression ratio of NKCC1/KCC2 occurs sooner in female than in male rats.<sup>37</sup> If similar differences in the developmental shift in expression ratio of NKCC1/KCC2 takes place in humans, neonate and infant male patients may be more prone to adverse effects of GABA<sub>A</sub>/glycine receptor enhancing anesthetics.

We hypothesize that despite the lack of efficacy of bumetanide, GABA<sub>A</sub>/glycine receptor-mediated inhibition produced by sevoflurane may play a role in the emergence seizures observed in rats with a mature neuronal Cl<sup>-</sup> gradient. In fact, we hypothesize that prolonged or enhanced inhibition produced by sevoflurane anesthesia *via* all mechanisms whereby sevoflurane acts, including sevoflurane-induced depression of glutamate release,<sup>11</sup> initiates compensatory increases in excitatory output to balance enhanced inhibition. Abrupt withdrawal of sevoflurane and decreased inhibition shift the inhibition or excitation balance toward greater excitation (*e.g.*, increased glutamate release and activation of *N*-methyl D-aspartate receptor and voltage-operated Ca<sup>2+</sup> channels). Increased intracellular Ca<sup>2+</sup> may increase inhibition of KCC2 and shift *E*<sub>Cl</sub> in mature neurons to more positive values.<sup>38</sup> Both increase in excitatory glutamatergic output and excitatory or less than normal inhibitory output of GABA and glycine systems because of inhibited activity of

KCC2 may provide a basis for the observed hyperexcitability phenomena during emergence. Furthermore, if  $E_{Cl}$  is indeed shifted to more positive values, agents with predominately GABA<sub>A</sub>/glycine receptor-mediated activity, such as benzodiazepines, may be ineffective in treating emergence hyperexcitability phenomena. In contrast, anesthetics with a predominately antigitamatergic action, such as ketamine, may in fact be the drugs of choice for treating emergence hyperexcitation. In support of these hypotheses are clinical data that anesthetics with anti-NMDA actions relieve emergence hyperexcitability in patients, whereas midazolam, which enhances GABAergic signals, does not.<sup>39,40</sup>

In addition to epileptic seizures and apoptosis, we found that sevoflurane produced both acute and long-term effects on LTP, which is considered a cellular correlate of memory formation and learning. In agreement with previously reported findings,<sup>11</sup> sevoflurane reversed LTP in brain hippocampal slices isolated from neonatal rats. Even more importantly, impaired LTP was recorded from hippocampal slices isolated from the brains of juvenile rats that were exposed to sevoflurane anesthesia during the early postneonatal period (fig. 6). In contrast to epileptogenic and neurotoxic effects of sevoflurane, pretreatment of neonatal rats with bumetanide before sevoflurane exposure did not significantly affect sevoflurane-induced impairment of LTP induction in hippocampal slices isolated from brains of juvenile rats. This finding indicates that sevoflurane may also cause adverse effects *via* other, NKCC1-independent, mechanisms.

In summary, the current results demonstrate that sevoflurane anesthesia may cause epileptic seizure activity, neurotoxicity, and acute as well as delayed impairment in synaptic plasticity in neonatal rats. The epileptogenic and neurotoxic effects of sevoflurane can be alleviated by the NKCC1 inhibitor bumetanide. On the other hand, the emergence seizures in older rat pups observed after termination of sevoflurane anesthesia and delayed impairment of synaptic plasticity could not be prevented by bumetanide. These findings suggest that inhibiting NKCC1 activity may be a useful approach to improve efficacy and safety of the anesthetics whose action involves potentiation of GABA<sub>A</sub>/glycine receptor function. These findings also justify further investigation of the mechanisms mediating these side effects of sevoflurane anesthesia.

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