Developmental Effects of Neonatal Isoflurane and Sevoflurane Exposure in Rats

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ABSTRACT

Background: The general anesthetics, isoflurane and sevoflurane, cause developmental abnormalities in neonatal animal models *via* incompletely understood mechanisms. Despite many common molecular targets, isoflurane and sevoflurane exhibit substantial differences in their actions. The authors sought to determine whether these differences can also be detected at the level of neurodevelopmental effects.

Methods: Postnatal rats, 4–6 days old, were exposed to 1.2% isoflurane or 2.1% sevoflurane for 1–6 h and studied for immediate and delayed effects.

Results: Isoflurane exposure was associated with weaker seizure-like electroencephalogram patterns than sevoflurane exposure. Confronted with a new environment at a juvenile age, the sevoflurane-exposed rats spent significantly more time in an "immobile" state than unexposed rats. Electroencephalographic (mean ± SE, 55.5 ± 12.80 s *vs.* 14.86 ± 7.03 s; $P = 0.014$; n = 6–7) and spontaneous behavior ($F_{(2,39)} = 4.43$; $P = 0.018$) effects of sevoflurane were significantly diminished by pretreatment with the Na^+–K^+–$2Cl^-$ cotransporter inhibitor bumetanide, whereas those of isoflurane were not. Pretreatment with bumetanide, however, diminished isoflurane-induced activation of caspase-3 in the cerebral cortex ($F_{(2,8)} = 22.869$; $P = 0.002$) and prevented impairment in sensorimotor gating function ($F_{(2,36)} = 5.978$; $P = 0.006$).

Conclusions: These findings in combination with results previously reported by the authors suggest that isoflurane and sevoflurane produce developmental effects acting *via* similar mechanisms that involve an anesthetic-induced increase in neuronal activity. At the same time, differences in their effects suggest differences in the mediating mechanisms and in their relative safety profile for neonatal anesthesia.

What We Already Know about This Topic

- Whether early postnatal exposure to volatile anesthetics produces long-term neurodevelopmental abnormalities in children remains a matter of debate.
- Sevoflurane and isoflurane, despite acting *via* common cellular mechanisms, exhibit substantial differences in their actions.

What This Article Tells Us That Is New

- At subanesthetic concentrations isoflurane and sevoflurane produce developmental effects in neonatal rats acting *via* similar mechanisms that may involve an increase in neuronal activity. At the same time, substantial differences in the effects of the two drugs suggest differences in the mechanisms mediating their actions and in their safety profile for neonatal anesthesia.

OPERATIVE procedures for millions of preterm and sick babies with different pathophysiological conditions require general anesthesia and repeated exposures are often needed. Considering the immense brain plasticity during this period of life, and the profound effects of general anesthetics on almost all aspects of central nervous system function, it is not surprising that there is a high level of concern among professionals and knowledgeable parents that exposure of newborns to general anesthesia may alter the course of brain development.[1–3] Animal studies across various species, from rodents to nonhuman primates, demonstrate that volatile anesthetics, including isoflurane and sevoflurane, may cause profound neuronal death, if applied during early stages of postnatal brain development.[4–15] Many of these studies reported long-term neurocognitive abnormalities detected using different experimental paradigms,[4,7–9,13–15] though the link between neuronal death and delayed functional abnormalities remains unclear.[16] The results of human retrospective epidemiological studies are less conclusive. Four of nine such studies did not detect long-term developmental abnormalities in children who had general anesthesia at a young age.[17–24] It is obvious that, in addition to other

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factors, the design of human studies lacks focus because the mechanisms that mediate the adverse developmental effects of general anesthetics are incompletely understood even in animal models. It is plausible that some anesthetics are more harmful than others, just as some neonates may be more susceptible to neurodevelopmental problems, for example, if their diseases share mechanisms with the side effects of anesthetics. New studies will be needed to address these possibilities.

We have recently found that sevoflurane, administered to neonatal rats, not only causes brain function-related developmental effects, such as seizure-like electroencephalogram patterns, an increase in levels of activated caspase-3 in the cerebral cortex, and an impairment in sensorimotor gating function, measured as a decrease of the prepulse inhibition (PPI) of the acoustic startle response 3 weeks after exposure to anesthesia, but also a prominent increase in serum levels of aldosterone.[10,15] Aldosterone is a key component of the hypothalamic–pituitary–adrenal axis and the surgical stress response. Exogenous administration of aldosterone further aggravated developmental outcomes of neonatal anesthesia with sevoflurane.[15]

Sevoflurane and isoflurane, despite acting *via* common cellular mechanisms, such as an enhancement of γ-aminobutyric acid type A ($GABA_A$) receptor activity, and activation of two-pore domain potassium leak channels, exhibit substantial differences in their actions.[25] Among others, isoflurane inhibits a major component of the excitatory glutamatergic synaptic transmission, namely postsynaptic *N*-methyl-D-aspartate receptors,[26–28] whereas sevoflurane may not produce this effect.[29] The differences in the side effects of these two otherwise very similar anesthetics, with relatively well-studied mechanisms of action if detected, would provide further insight into the relative safety profile of the two anesthetics, and the mechanisms mediating the side effects of general anesthesia in neonates. Therefore, we compared acute and delayed effects of equipotent exposures with isoflurane and sevoflurane in a neonatal rat model.

Materials and Methods

Animals

All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee (Gainesville, FL). Sprague–Dawley rats 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 pups were determined as well nourished, judged by their stomachs being full of milk (detectable through the transparent abdominal wall). Different sets of animals were used in each given experiment.

Anesthesia and Electroencephalogram Recording

In order to study the effects of isoflurane and sevoflurane, anesthesia was induced in a temperature-controlled small chamber with 3.4% isoflurane (6% sevoflurane) and 1.5 l/min oxygen over 3 min, and maintained with 1.2% isoflurane (2.1% sevoflurane) and 1.5 l/min oxygen over 60 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.*,[30] 1.2% isoflurane and 2.1% sevoflurane lie near 0.6 minimum alveolar concentration for P4–P7 rats. At the doses of 2.1 % sevoflurane and 1.2% isoflurane, rat pups appeared to be fully anesthetized in the absence of surgical procedures. Blood glucose levels after 6 h of anesthesia with 1.2% isoflurane and 2.1% sevoflurane were 120.3 ± 3.7 (n = 6) and 125.3 ± 3.8 (n = 3), respectively. To study the effects of isoflurane and sevoflurane on cortical activity, rat pups ranging from postnatal days 4–6 (P4–P6) were instrumented for electroencephalogram recording, as described previously.[10,15] In brief, during a 12–15-min long minor surgical procedure, which was performed under isoflurane anesthesia (1.6–2.0%), four electrodes of the headmounts of the electroencephalogram /electromyogram system (Pinnacle Technology, Lawrence, KS) were implanted. No obvious differences in electroencephalographic activity were detected when electroencephalogram electrode implantation was done either immediately before, or 1 or 2 days before start of electroencephalogram recording. Electroencephalogram patterns, characterized by an amplitude at least three times higher than the baseline and rhythmic (>2 Hz) activity, which lasted for at least 3 s and abruptly reverted to baseline, were defined as seizure-like electroencephalogram patterns.[31] In most cases, these patterns started as high-frequency, low-amplitude activity, developed to increased amplitude and decreased frequency, and then abruptly reverted to baseline activity. The seizure-like electroencephalogram patterns were detected visually by at least three independent reviewers, and a consensus was reached for summary data. Animals that exhibited episode(s) of seizure-like electroencephalogram patterns before the start of anesthesia were not included in the data analysis.

Determination of Activated Cleaved Caspase-3 Using Western Blot

The levels of activated caspase-3 in the cerebral cortex were determined as described previously.[10,15] Western blot analysis for tissue samples from each animal was done in triplicate and reported as an average.

Measurements of Acoustic Startle Response and PPI of Startle

The PPI of startle tests were performed, using the SR-Lab startle apparatus (San Diego Instruments, San Diego, CA) as previously described by our laboratory.[15] The %PPI for each prepulse intensity was calculated using the following formula: %PPI = 100 × ([pulse alone] – [prepulse + pulse])/pulse alone.[32] Data were collected as average amplitude of the 1,000 ms-long recording window.

Immobility Behavior Testing

Increased immobility in rodents is used as behavioral test for anxiety and depression.[33] The rats were video-recorded in a homemade clear plexiglas chamber (28 cm diameter/30.5 cm height). Each rat was placed in the chamber alone during the video recording. A camera was focused on the rat, providing a close-up view of the rat's body. The behavior of each rat was analyzed during a period of 10 min. Immobility was defined as a sudden pause of all locomotion lasting at least 10 s. Termination of immobility was defined as the consecutive movement of the two front paws in a walking manner. The data are presented as total time spent in an immobile state.

Drugs

Isoflurane and sevoflurane were manufactured by Minrad Inc. (Bethlehem, PA) and Fushimi-machi (Osaka, Japan), respectively. Bumetanide (Ben Venue Laboratories, Inc.) was purchased from Bedford Laboratories™ (Bedford, OH). Cleaved caspase-3 antibodies were acquired from (Cell Signalling, Danvers, MA), and horseradish peroxidase-conjugated goat antirabbit and anti- γ-tubulin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Statistical Analysis

Values are reported as mean ± SEM. SigmaStat 3.11 software (Systat Software Inc., Point Richmond, CA) was used for statistical analyses. Single comparisons were tested using the unpaired *t* test, whereas multiple comparisons among groups were analyzed using one-way ANOVA, followed by Holm–Sidak tests. Changes in PPI of startle for three prepulse intensities in multiple groups were analyzed using two-way repeated-measures ANOVA, followed by Holm–Sidak tests. All comparisons were run as two-tailed tests. A *P* value less than 0.05 was considered significant.

Results

Anesthesia with isoflurane, in contrast to anesthesia with sevoflurane, is associated with few seizure-like electroencephalogram patterns, which were not further diminished by pretreatment with bumetanide. Electroencephalographic activity in postnatal days 4–6 (P4–P6) rats was recorded during two 1-h periods: 1 h baseline before, and then 1 h during anesthesia, with isoflurane or sevoflurane (fig. 1A). Fifteen min before initiating the anesthetic, half of the rats were treated with bumetanide (5 μmol/kg, intraperitoneal) and the other half received an equal volume of saline. During the 3-min induction period with 3.4% isoflurane, one of eight rats pretreated with saline had a 20 s-long episode of electroencephalographic activity, which met our criteria for a seizure-like electroencephalogram pattern. During the 57-min maintenance period with 1.2% isoflurane, seizure-like electroencephalogram patterns in this treatment group were detected in five of seven rats (fig. 1, B–D). This electroencephalogram-detectable seizure-like activity consisted of 1.9 ± 0.8 episodes, with single episodes lasting 9.2 ± 2.1 s and total durations of 21.9 ± 9.3 s. All analyzed parameters of seizure-like electroencephalogram patterns, *e.g.,* total duration, number of episodes, and single episode duration, tended to decrease in rats pretreated with bumetanide; however, these effects of bumetanide were not sufficient to achieve statistical significance (fig. 1B). We compared the effects of bumetanide on sevoflurane-caused electroencephalogram-detectable hyperexcitation events in an experiment of the same design, *i.e.,* equipotent concentration of sevoflurane administered to the P4–P6 rats for the same duration of time. The seizure-like activity caused by sevoflurane was more pronounced (fig. 1E); rats pretreated with bumetanide had significantly shorter total durations of

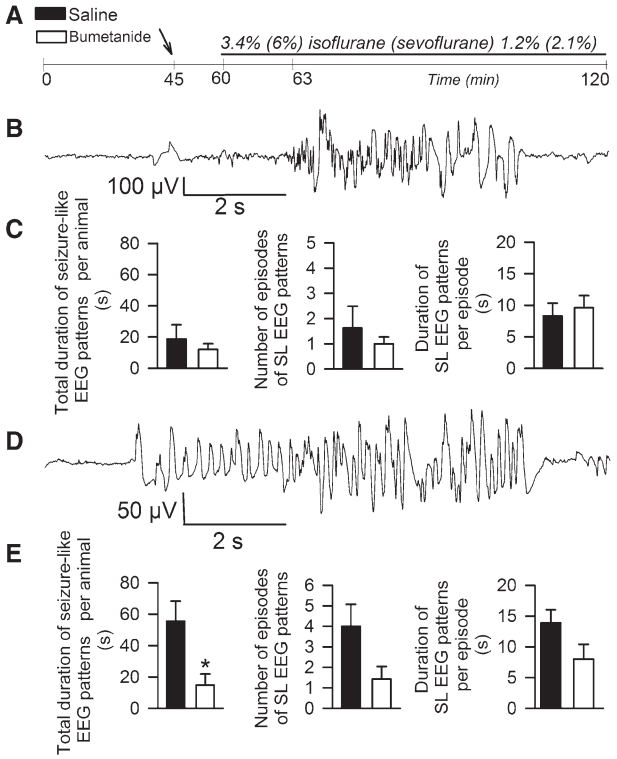

Fig. 1. Seizure-like electroencephalogram (EEG) patterns in neonatal rats anesthetized with isoflurane or sevoflurane. *A*, Illustration of the experimental protocol. *B*, An example of electroencephalogram recording of seizure-like electroencephalogram pattern in a P6 rat during 1.2% isoflurane anesthesia. *C*, Histograms showing properties of seizure-like electroencephalogram (SL EEG) patterns in neonatal rats anesthetized with 1.2% isoflurane that were pretreated with either bumetanide (5 μmol/kg, intraperitoneally, n = 8), or equal volume of saline (n = 8). *D*, An example of electroencephalogram recording of seizure-like electroencephalogram pattern in a P6 rat during 2.1% sevoflurane anesthesia. *E*, Histograms showing properties of seizure-like electroencephalogram patterns in neonatal rats anesthetized with 2.1% sevoflurane that were pretreated with either bumetanide (5 μmol/kg, intraperitoneally, n = 7), or equal volume of saline (n = 6). * $P < 0.05$ *versus* total duration of seizure-like electroencephalogram patterns in the saline-pretreated rats.

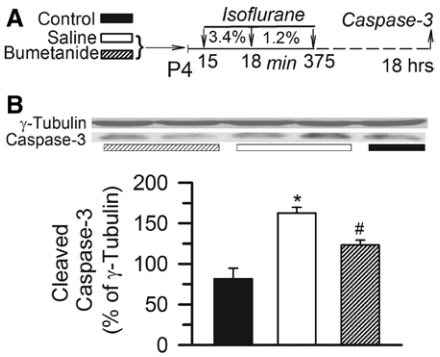

Fig. 2. Increased levels of activated caspase-3 in the cerebral cortex of neonatal rats that were anesthetized with isoflurane. *A*, Illustration of the experimental protocol. *B*, Representative Western blot images of cleaved caspase-3 and γ-tubulin blots and histogram showing results of the densitometric analysis of cleaved caspase-3 in the cortex tissue from three experimental groups. Rats in the two treatment groups were exposed to isoflurane anesthesia after they were pretreated with either bumetanide (5 μmol/kg, intraperitoneally, n = 3), or equal volume of saline (n = 3). Rats in the control group (n = 3) did not undergo anesthesia on postnatal day 4. Densities of γ-tubulin blots from the same tissue sample were taken as 100%. * P = 0.002 *versus* control and bumetanide, # P = 0.025 *versus* control.

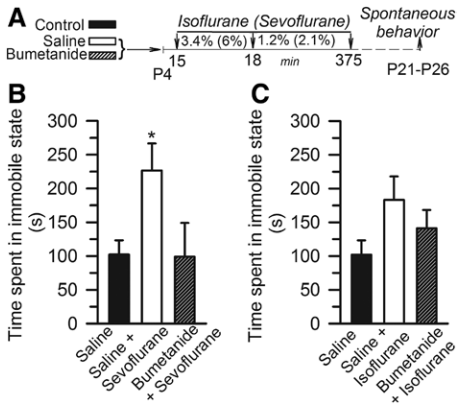

Fig. 3. Rats that were anesthetized with sevoflurane (but not isoflurane) on postnatal days 4 and 5 (P4 and P5), spent significantly increased time in immobile state; an effect that was alleviated by pretreatment with bumetanide. *A*, Illustration of the experimental protocols. *B,* The effect of sevoflurane. Histogram showing time spent in immobile state by three treatment groups: (1) control (n = 19), saline + sevoflurane (n = 13), and bumetanide + sevoflurane (n = 10). * P < 0.05 *versus* control. *C,* The effect of isoflurane. Histogram showing time spent in immobile state in three experimental groups: Rats in the two treatment groups were exposed to isoflurane anesthesia after they were pretreated with either bumetanide (5 μmol/kg, intraperitoneally, n = 12 per), or equal volume of saline (n = 12). Rats in the control group (n = 19) did not undergo anesthesia at P4. All nonanesthetized rats tested in spontaneous behavior experiments were combined in the control groups.

seizure-like electroencephalogram patterns during the 1-h exposure to sevoflurane, compared with rats that received saline before exposure to sevoflurane.

Isoflurane increases levels of activated caspase-3 in the cerebral cortex, an effect partially diminished by pretreatment with bumetanide.

As in our recent published study with sevoflurane,[15] in this study too, Western blot analysis revealed increased levels of activated caspase-3 in the cerebral cortex of P5 rats exposed to 6 h of anesthesia with isoflurane 1 day earlier. The rats that were pretreated with bumetanide (5 μmol/kg, ip) before exposure to isoflurane had lower levels of activated caspase-3 in comparison with those pretreated with saline and anesthetized with isoflurane ($F_{(2,8)}$ = 22.869; P = 0.002 *vs.* control and bumetanide groups; fig. 2, A and B). Pretreatment with bumetanide before anesthesia with isoflurane did not completely prevent the increase in levels of activated caspase-3 caused by isoflurane (P = 0.025 *vs.* control).

Anesthesia of neonatal rats with sevoflurane (but not isoflurane) results in bumetanide-sensitive delayed "immobile state" behavior. The effects of neonatal anesthesia, with isoflurane and sevoflurane at P4 and P5, on the subsequent spontaneous behavior in rats was tested when the animals achieved 21–26 days of age. Most of the rats were 24 days of age at the time of testing. When exposed to a new environment, rats previously anesthetized with sevoflurane spent a significantly longer time in an immobile state compared with nonanesthetized controls ($F_{(2,39)}$ = 4.43; P = 0.018 *vs.* control and bumetanide groups; fig. 3, A and B). No significant differences were observed between the control rats not exposed to anesthesia and those pretreated with bumetanide before exposure to sevoflurane at P4 and P5 (P = 0. c). Rats anesthetized with isoflurane at an early postnatal age also tended to spend more time in an immobile state, when compared with rats that were never exposed to anesthesia, or with those pretreated with bumetanide before exposure to isoflurane. However, the effects of neither isoflurane nor bumetanide were sufficient to achieve statistical significance ($F_{(2,40)}$ = 2.397; P = 0.104; fig. 3, A and C).

Isoflurane causes a long-term decrease of PPI of startle in control but not in bumetanide-pretreated rat pups. Startle stimuli by themselves caused similar responses in all three treatment groups. Startle response amplitudes were 15.8 ± 1.2, 15.2 ± 1.2, and 16.9 ± 1.4 in nonanesthetized, anesthetized, and bumetanide-pretreated anesthetized animals, respectively (fig. 4A; $F_{(2,36)}$ = 0.506; P = 0.607; one-way ANOVA). Startle stimuli, presented in combination with preceding prepulse stimuli, inhibited startle responses, but PPI of startle was less in animals exposed to isoflurane early after birth. Two-way repeated measures ANOVA analysis of PPI of startle found significant effects of prepulse intensities (main effect, $F_{(2,4)}$ = 118.287; P < 0.001), and treatment with isoflurane (main effect, $F_{(2,36)}$ = 5.978; P = 0.006; fig. 4B). The rats that were exposed to isoflurane at P4 exhibited reduced PPI of startle at all prepulse intensities when compared with nonanesthetized animals, although a significant

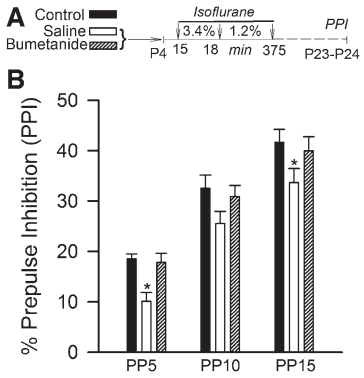

Fig. 4. Isoflurane anesthesia on postnatal days 4 and 5 (P4 and P5) causes abnormalities in prepulse inhibition (PPI) of the acoustic startle response evaluated at P23 and P24. Bumetanide (5 μmol/kg, intraperitoneally), administered before anesthesia, alleviated the effect of isoflurane on PPI of acoustic startle. *A*, Illustration of the experimental protocol. Rats in the control groups did not undergo anesthesia on P4 and P5. *B*, Histogram showing %PPI of startle in different treatment groups: control (n = 11), saline + isoflurane (n = 14), and bumetanide + isoflurane (n = 15). * $P < 0.05$ *versus* control. PP5–PP15, prepulse intensities in dB above background.

reduction was detected at prepulse intensities of 5 dB and 15 dB, but not of 10 dB. The effect of treatment did not depend on the prepulse intensities ($P = 0.980$). There was no significant reduction in PPI of startle in animals that received bumetanide (5 μmol/kg, intraperitoneal) before isoflurane anesthesia ($P = 0.571$ *vs.* nonanesthetized and $P = 0.011$ *vs.* isoflurane). We have previously demonstrated that a single dose of bumetanide alone without anesthesia did not result in any obvious changes in the acoustic startle response.[15]

Discussion

We have found that in neonatal rats isoflurane, similar to previously reported effects of sevoflurane,[15] caused a number of both acute and delayed adverse effects: seizure-like electroencephalogram patterns, neuroapoptosis in the cerebral cortex (measured as increased levels of activated caspase-3), and impairment of sensorimotor gating function. The latter could be detected weeks after exposure to the anesthetic. Our results also indicate that the effects of isoflurane and sevoflurane show important differences. Isoflurane-caused less pronounced seizure-like electroencephalogram patterns and nonsignificant increases in "immobility" behavior. Furthermore, bumetanide, the Na^+–K^+–$2Cl^-$ co-transporter inhibitor, did not alter the effects of isoflurane on electroencephalographic activity in rats. Pretreatment with bumetanide, however, decreased the isoflurane-caused impairment of PPI of the startle response, as previously seen in the sevoflurane-anesthetized animals.[15] Bumetanide partially diminished an increase in the levels of activated caspase-3 in the cerebral cortex, caused by isoflurane.

Seizure-like electroencephalogram patterns detected during anesthesia of neonatal rats with sevoflurane and isoflurane indicate that the anesthetics may increase neuronal cortical activity at this age. These findings, taken together with the effects of bumetanide in the isoflurane- and sevoflurane-anesthetized neonatal rats, suggest that the anesthetic-caused apoptotic cell death and long-term functional abnormalities (impairment of sensorimotor gating function and increase in immobility patterns in rats previously anesthetized with sevoflurane) result, at least in part, from the anesthetic-caused increase of neuronal activity. Importantly, our findings do not link the anesthetic-caused seizure-like electroencephalogram patterns as prerequisites to neuronal death and functional effects, as well as neuronal death to delayed functional abnormalities. Although current understanding of the developmental neurophysiology, mechanisms of action of volatile anesthetics, and neuronal effects of bumetanide makes $GABA_A$ receptor-mediated signaling the most plausible candidate for mediating the anesthetic-induced neuronal stimulation in the developing brain, the results of our experiments with bumetanide do not exclude other potential mechanism(s) contributing to the anesthetic-caused increase of neuronal activity in the developing brain. Our pilot studies suggest that the anesthetic-activated hypothalamic–pituitary–adrenal axis may represent one of such mechanisms. It is plausible that the anesthetic-increased neuronal activity to certain level, independent of the mediating mechanisms, is sufficient for the observed side effects to occur. Bumetanide, by decreasing $GABA_A$ receptor-mediated excitation or promoting $GABA_A$ receptor-mediated inhibition, decreases total neuronal activity in the developing brain and alleviates the anesthetic-caused side effects. In line with this, less pronounced seizure-like electroencephalogram patterns caused by isoflurane may be the result of less neuronal stimulation by isoflurane due to inhibitory actions of the anesthetic *via* additional pathways that are not engaged by sevoflurane. For example, sevoflurane may be less potent than isoflurane at inhibition of *N*-methyl-D-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subtype of postsynaptic glutamate receptors[26–28] or may even not have any effect on functioning of these receptors.[29] The inhibitory profile of isoflurane is further supported by its depression of presynaptic glutamate release.[30] Still, the alleviating effects of bumetanide on isoflurane-caused increases in levels of activated caspase-3 and impairment in sensorimotor gating function, suggest a role of the anesthetic-caused neuronal stimulation in its developmental effects. Previously, Sanders *et al.*[34] observed cell death in mouse organotypic hippocampal slice cultures exposed to a combination of 0.75% isoflurane and 50 μM $GABA_A$ receptor antagonist SR95531 (gabazine), calling into question the involvement of $GABA_A$ receptor mediated-signaling in the neurotoxic effects of isoflurane. Given that slice cultures with substantially altered or eliminated extrinsic inputs or outputs and hormonal or neurotransmitter controls represent a simplified model of the *in vivo* environment, it is difficult to directly compare our *in vivo* measurements

with bumetanide and the measurements with gabazine in slice cultures.[34] Nonetheless, the results with gabazine may support our hypothesis on the role of increased neuronal activity in the adverse effects of isoflurane and sevoflurane in the developing brain, because treatment of P10 and P11 hippocampal slices with gabazine in the presence of isoflurane is likely to increase neuronal activity, though gabazine alone in the absence of the anesthetic did not cause any effect.

A possibility that isoflurane may cause alterations in the sensorimotor gating function in neonatal rats by acting at $GABA_A$ receptors is indirectly supported by the findings reported by other laboratories that phenobarbital and allopregnanolone, both positive $GABA_A$ receptor modulators, caused long-term impairment of PPI of startle after single administration to neonatal rats.[35,36] A recent study found that reduction of PPI of startle in juvenile subjects may be a predictor of predisposition to schizophrenia before other symptoms of the illness can be detected.[37] Sensorimotor gating deficits are characteristic of a number of other human neuropsychiatric diseases,[38,39] raising the possibility that subjects predisposed to these disorders may be more susceptible to the developmental effects of isoflurane and sevoflurane.

In summary, these findings, in combination with our previous reports,[10,15] suggest that isoflurane and sevoflurane may produce developmental effects acting *via* similar mechanisms involving an increase in neuronal activity. At the same time, substantial differences in the effects of isoflurane and sevoflurane in neonatal rats suggest differences in the mechanisms mediating their actions, and more importantly, also potentially on the relative safety profile of the two anesthetics for neonatal anesthesia. If these findings translate to humans then by extension isoflurane may be preferred over sevoflurane as an anesthetic for very young pediatric patients, especially to those predisposed to epileptic seizures.

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