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Early Exposure to General Anesthesia with Isoflurane Downregulates Inhibitory Synaptic Neurotransmission in the Rat Thalamus

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Abstract Recent evidence supports the idea that common general anesthetics (GAs) such as isoflurane (Iso) and nitrous oxide (N_2O; laughing gas) are neurotoxic and may harm the developing mammalian brain, including the thalamus; however, to date very little is known about how developmental exposure to GAs may affect synaptic transmission in the thalamus which, in turn, controls the function of thalamocortical circuitry. To address this issue we used in vitro patch-clamp recordings of evoked inhibitory postsynaptic currents (eIPSCs) from intact neurons of the nucleus reticularis thalami (nRT) in brain slices from rat pups (postnatal age P10–P18) exposed at age of P7 to clinically relevant GA combinations of Iso and N_2O. We found that rats exposed to a combination of 0.75 % Iso and 75 % N_2O display lasting reduction in the amplitude and faster decays of eIPSCs. Exposure to sub-anesthetic concentrations of 75 % N_2O alone or 0.75 % Iso alone at P7 did not affect the amplitude of eIPSCs; however, Iso alone, but not N_2O, significantly accelerated decay of eIPSCs. Anesthesia with 1.5 % Iso alone decreased amplitudes, caused faster decay and decreased the paired-pulse ratio of eIPSCs. We conclude that anesthesia at P7 with Iso alone or in combination with N_2O causes plasticity of eIPSCs in nRT neurons by both presynaptic and postsynaptic mechanisms. We hypothesize that changes in inhibitory synaptic transmission in the thalamus induced by GAs may contribute to altered neuronal excitability and consequently abnormal thalamocortical oscillations later in life.

Keywords Nitrous oxide · Isoflurane · $GABA_A$ receptor · GABAergic interneuron · Brain development · Synaptogenesis

Introduction

Most currently used GA agents have either *N*-methyl-D-aspartate (NMDA) receptor-blocking or/and γ-aminobutyric acid A ($GABA_A$) receptor-enhancing properties, which are thought to be essential for their sedative/hypnotic properties [1]. Unfortunately, it has been well documented that increased activation of $GABA_A$ receptors and/or blockade of NMDA receptors can trigger widespread neurodegeneration in developing rodent and non-human primate brains including in the thalamus [2–6]. Although human studies addressing the issue of safety of clinical anesthesia in the developing brain still are not conclusive, at least some concerns have been raised [7]. Further research is warranted to elucidate cellular mechanisms for long-lasting effects of currently available GAs on neuronal function and to develop possible therapeutic strategies that could be used to make clinical anesthesia practice safer.

Thalamic nuclei are strongly implicated in awareness, cognitive functions, memory, sleep and wake cycles [8–11]. Both human and animal studies in vivo have established that the thalamus is deactivated during acute application of GAs [11, 12]. We have previously demonstrated that acute effects of Iso on vesicular release of GABA in the nucleus reticularis thalami (nRT) likely contribute to its useful clinical effects such as sedation and hypnosis [13]. In another recent study, we showed that exposure of rat pups at the age of P7 to a clinically

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relevant anesthetic cocktail consisting of 0.75 % Iso, 75 % N_2O and 9 mg/kg of midazolam triggers lasting plasticity of synaptic (both inhibitory and excitatory) and intrinsic ion channels such as T-type calcium channels (T-channels) in neurons of the nRT. This plasticity, in turn, contributes to lasting hyperexcitability in mutually connected cortical and thalamic sensory neurons (thalamocortical networks), as demonstrated using both in vitro and in vivo methods such as patch-clamp recordings and electroencephalography (EEG), respectively [14]. However, our previous study did not address individual contributions of these drugs to the plasticity of ion channels in nRT neurons. Here, we used patch-clamp recordings in intact brain slices to address the question whether Iso and N_2O alone and in combination can cause any lasting alterations of inhibitory synaptic transmission as assessed by properties of eIPSCs mediated by $GABA_A$ receptors.

Material and Methods

Anesthesia At postnatal day 7 (P7) both male and female Sprague Dawley rats were exposed to 6 h of clinically relevant concentrations of GAs with 75 % N_2O plus 0.75 % Iso in combination, 75 % N_2O alone, 0.75 % Iso alone or 1.5 % Iso alone (Fig. 1). Typically, sham controls were littermates exposed to 6 h of mock anesthesia consisting of separation from their mother in an air-filled chamber. An agent-specific vaporizer was used to deliver a set percentage of Iso with a mixture of O_2 and N_2O gases into a temperature-controlled chamber preset to maintain 33–34 °C. The composition of the gas chamber was analyzed using real time feedback (Datex Capnomac Ultima) for N_2O, Iso, CO_2, and O_2 percentages.

Brain Slice Preparation Details of our experimental protocols were described previously [13, 14]. Most experiments were done on transverse rat brain slices 250–300 μm thick taken through the middle anterior portion of the nRT [15]. Sprague–Dawley rats were housed in the local animal facility in accordance with protocols approved by the University of

Fig. 1 Scheme depicts timeline of events in our experimental procedures

Virginia Animal Use and Care Committee. All treatment of rats adhered to the guidelines in the *NIH Guide for the Care and Use of Laboratory Animals*.

Young rats (P10-P18) were deeply anesthetized with Iso and decapitated. The brains were removed rapidly and placed in chilled (4°C) cutting solution consisting, in millimolar, of 2 $CaCl_2$, 260 sucrose, 26 $NaHCO_3$, 10 glucose, 3 KCl, 1.25 NaH_2PO_4, and 2 $MgCl_2$ equilibrated with a mixture of 95 % O_2 and 5 % CO_2. A block of tissue containing the thalamus was glued to the chuck of a vibrotome (WPI, Saratoga, FL) and 250–300-μm slices were cut in a transverse plane. The slices were incubated in 36 °C oxygenated incubation solution for 1 h, then placed in a recording chamber that had been superfused with extracellular saline at a rate of 1.5 cc/min. Incubation solution consisted, in millimolar, of 124 NaCl, 4 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $MgCl_2$, 10 glucose, and 2 $CaCl_2$ equilibrated with a mixture of 95 % O_2 and 5 % CO_2. Slices were maintained in the recording chamber at room temperature and remained viable for at least 1 h. Since the half-life of halogenated volatile anesthetics in nerve tissue after induction of anesthesia is only about 10 min [16], it is unlikely that the brief Iso exposure used to euthanize animals could have interfered with the results of our experiments, which were performed at least 2 h later.

Recording Procedures The standard extracellular solution for recording of eIPSCs consisted in mM, of 2 $CaCl_2$, 130 NaCl, 1 $MgCl_2$, 10 glucose, 26 $NaHCO_3$, 1.25 NaH_2PO_4, and 2 mM KCl. For recordings of eIPSCs, we used an internal solution containing, in millimolar, 130 KCl, 4 NaCl, 0.5 $CaCl_2$, 5 EGTA, 10 HEPES, 2 $MgATP_2$, 0.5 Tris-GTP, and 5 lidocaine *N*-ethyl bromide (QX-314). To eliminate glutamatergic excitatory currents, all recordings of eIPSCs were done in the presence of 5 μM 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) and 50 μM (2*R*)-amino-5-phosphonovaleric acid (d-APV). All experiments were done at room temperature (20–24 °C). Whole-cell recordings were obtained from nRT neurons visualized with an infrared (IR) DIC camera (Hammamatsu, C2400) on a Zeiss 2 FS Axioscope (Carl Zeiss, Jena) with a ×40 lens.

Electrophysiological Recordings Synaptic stimulation of nRT neurons was achieved with a Constant Current Isolated Stimulator DS3 (Digitimer Ltd., Welwyn Garden City, Hertfordshire, England) and electrical field stimulation was achieved by placing a stimulating electrode in the outer region of the internal capsule [13]. Recordings were made with standard whole cell voltage clamp technique. Electrodes were fabricated from thin-walled microcapillary glass with a final resistance of 3–6 MΩ. Membrane currents were recorded with an Axoclamp 200B amplifier (Molecular Devices, Foster City, CA). Voltage commands and digitization of membrane currents were done with Clampex 8.2 of the pClamp software
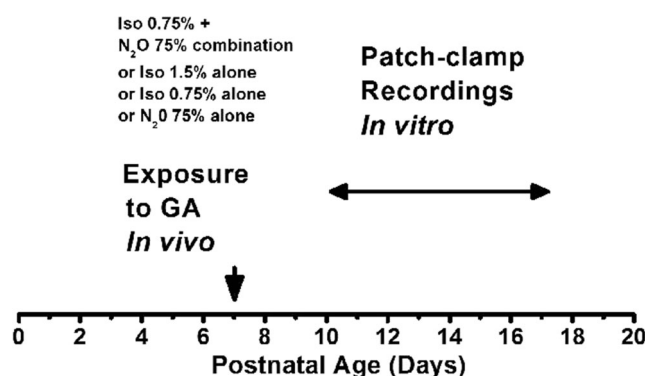
package (Molecular Devices) running on an IBM-compatible computer. Neurons typically were held at −70 mV. Currents were filtered at 5–10 kHz. Series resistance typically was compensated by 50–80 % during experiments.

Analysis of Current Current waveforms or extracted data were fit using Clampfit 8.2 (Molecular Devices) and Origin 7.0 (OriginLab, Northhampton, MA). The decay time constant (decay τ) of eIPSCs was estimated by a single- or double-exponential term. If double exponential function was required, we used weighted averages for our analyses.

Statistical analysis was done with two-tailed Student's t test and Mann–Whitney Rank Sum test where indicated, with statistical significance determined at $p < 0.05$. Input–output curves were analyzed using two-way ANOVA with repeated measures followed with Tukey HSD multiple comparisons.

Drugs and Chemicals Isoflurane was obtained from Abbott (Abbott Park, IL). All other salts and chemicals were obtained from Sigma Chemical (St. Louis, MO). All drugs were prepared as stock solutions and were diluted freshly to the appropriate concentrations at the time of experiments. All stocks were prepared in sterile water except for NBQX and picrotoxin, which were prepared in dimethyl sulfoxide (DMSO). The maximum final concentration of DMSO in any one experiment was 0.1 %; at that concentration, DMSO has no effect on eIPSCs in nRT cells ($n = 3$, data not shown).

Results

Electrical stimulation of fibers in close proximity to the internal capsule (approximately 200 μm from the recorded nRT neurons) evokes picrotoxin-sensitive $GABA_A$-mediated eIPSCs as we described previously [13]. Figure 2 (panel A) shows a family of traces of eIPSCs evoked by a series of escalating stimuli that were used to generate input–output (I–O) curves in a representative nRT cell from a rat treated with a combination of 0.75 % Iso and 75 % N_2O. In these experiments, we first determined threshold stimulus (denoted as 1) in each experiment, then we used progressively stronger stimuli 1-, 2-, 3-, 4-, and 5-fold higher than the threshold stimulus. We next investigated whether a single 6-h-long exposure to 0.75 % Iso or 75 % N_2O alone or in combination might affect the I–O curves of eIPSCs. A bar graph depicting results of the stimulus I–O curves from multiple experiments (Fig. 2b) reveals eIPSCs amplitude depression across stimulus intensities with the 0.75 % Iso and 75 % N_2O combination (black bar, $n = 11$ neurons) as compared with the sham group (white bar, 23 neurons, *indicates $p < 0.05$ and **indicates $p < 0.01$, two-way ANOVA followed with Tukey multiple
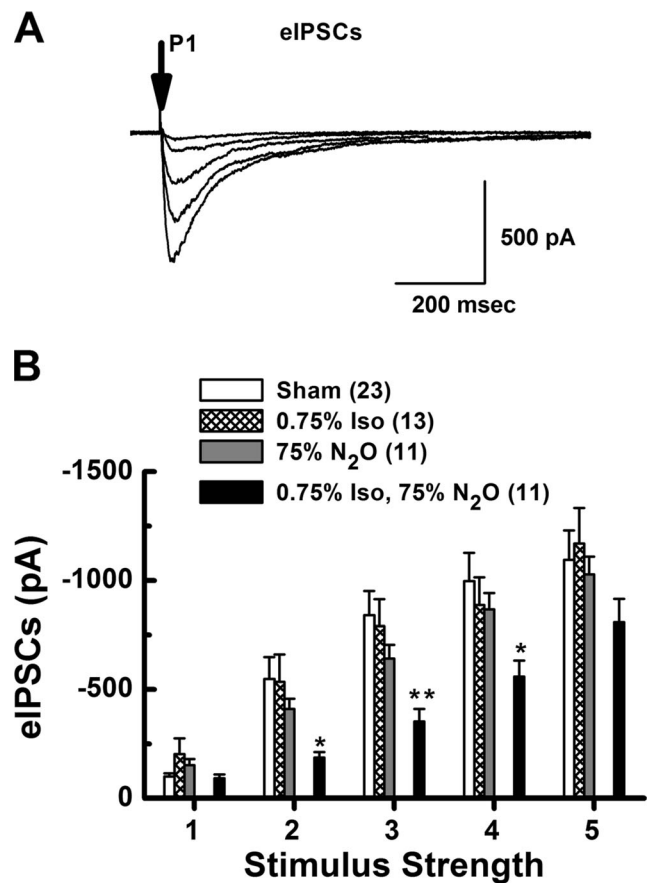

Fig. 2 Decreased synaptic strength of eIPSCs in rat pups exposed to the combination of 0.75 % Iso and 75 % N_2O. **a** Panel depicts family of inward eIPSCs evoked with escalating amplitudes of pulse stimuli (P1) in a representative nRT neuron from GA-treated rat. **b** Average I–O curves from multiple experiments are plotted. *Numbers in parenthesis* indicate number of neurons in each condition

comparisons). Neither 0.75 % Iso alone (crossed bar, $n = 13$ neurons) nor 75 % N_2O alone (gray bar, $n = 11$ neurons) had significant effect on the amplitudes of eIPSCs as compared with those from nRT neurons in the sham group. Next, we used paired-pulse analysis as a sensitive method to discern pre-synaptic vs. post-synaptic mechanisms of synaptic plasticity. In nRT neurons, stimulation with a paired-pulse stimulus interval of 0.01–10 s usually results in depression of the second (test) eIPSC as compared with the first (conditioning) eIPSC (Fig. 3a). This depression of paired-pulse ratio (PPR) of test IPSCs (P2) relative to conditioning IPSCs (P1) is thought to be due to depletion of a fraction of readily available synaptic vesicles by the conditioning pulse [17]. The bar graph in Fig. 3b shows that the average PPR was slightly decreased in exposed pups (black bar, 0.76 ± 0.04, $n = 13$ neurons) as compared with that in sham controls (white bar, 0.85 ± 0.02, $n = 47$ neurons) but it did not reach statistical significance ($p = 0.07$, Mann–Whitney Rank Sum test). Furthermore, the bar graph in Fig. 3c demonstrates that the average decay τ for eIPSCs in the same cells from exposed pups (black bar, 88
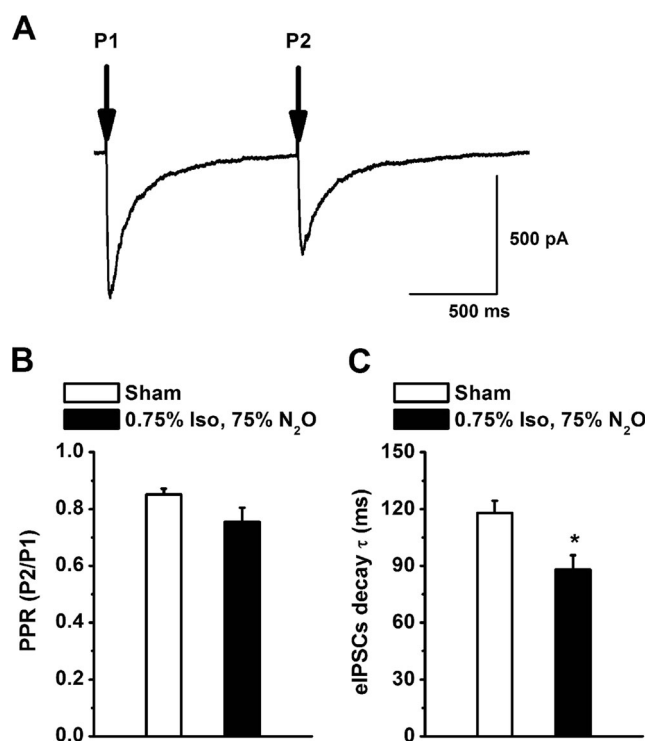
Fig. 3 Plasticity of eIPSCs in rat pups exposed to the combination of 0.75 % Iso and 75 % N$_2$O. **a** Paired-pulse stimulus protocol shows two stimuli (designated as P1 and P2) separated by 1 s and the trace of resulting eIPSC in a representative nRT neuron from a rat exposed to the anesthetic double combination. **b** *Bar graph* shows that in GA-treated animals, the average PPR ratio is not significantly decreased as compared with that in sham-treated animals. **c** Bar graph shows that in GA-treated animals, the average decay τ of eIPSCs is significantly decreased about 25 % as compared with that in sham-treated animals

±8 ms) was significantly accelerated as compared with that from sham controls (white bar, 118±6 ms, *p*=0.02, *t* test).

Finally, we examined the effects of exposure of young pups to sub-anesthetic concentrations (0.75 %) and anesthetic concentrations (1.5 %) of Iso alone. Figure 4a shows average traces of eIPSCs from the sham group (black trace) and the experimental groups exposed either to 0.75 % Iso (gray trace) or to 1.5 % Iso (red trace). Note that 0.75 % Iso had very little effect on the amplitude of eIPSCs, while 1.5 % Iso reduced the amplitude of eIPSCs by about 60 %. In contrast, Fig. 4b shows that both concentrations of 0.75 and 1.5 % Iso significantly (*, *p*=0.02, *t* test) reduced the average decay τ of eIPSCs as compared with that from sham control (black bar, control, 118± 6 ms, *n*=47 neurons) to 85±5 ms (gray bar, 0.75 % Iso, *n*=13 neurons) or 83±8 ms (red bar, 1.5 % Iso, *n*=11 neurons). Next, we performed a double-pulse experiment to compare PPR between sham group and the group exposed to 1.5 % Iso. The bar graphs in Fig. 4c show that the average PPR was significantly decreased (*, *p*<0.02 by Mann–Whitney Rank sum test)
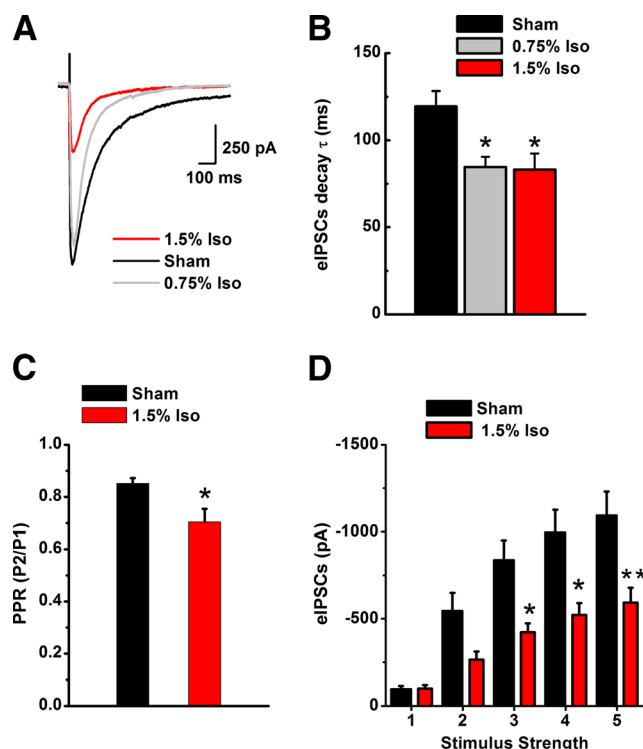
Fig. 4 Plasticity and decreased synaptic strength of eIPSCs in rat pups exposed to anesthesia with 0.75 % Iso or 1.5 % Iso alone. **a** This panel depicts average traces from nRT neurons from the sham group (black trace, 24 neurons) and the GA-exposed groups with 0.75 % Iso (*gray trace*, 13 neurons) and 1.5 % Iso (*red trace*, 11 neurons). Note that only 1.5 % Iso robustly decreased the amplitude of eIPSCs; but faster decay τs of eIPSCs are evident in both GA groups. **b** The average decay time constants of eIPSCs are decreased significantly in the nRT neurons from both GA groups (*gray bar*—0.75 % Iso; *red bar*—1.5 % Iso) as compared with the nRT neurons from sham controls (*black bar*). **c** *Bar graph* shows that in nRT neurons from 1.5 % Iso-treated animals (*red bar*) the average PPR ratio is decreased significantly as compared with the nRT neurons from sham-treated animals (*black bar*). **d** The averaged data from I-O curves show decreased synaptic strength of eIPSCs across the stimuli in the GA group treated with 1.5 % Iso (*red bars*) versus the sham group (*black bars*)

in the Iso group (red bar, 0.70±0.05, *n*=7 neurons) as compared with that from the sham group (black bar, 0.85±0.02, *n*=47 neurons).

We also examined synaptic strength of eIPSCs in the experimental group exposed to 1.5 % Iso using I–O curves. The averaged data from I–O curves (Fig. 4d) show decreased synaptic strength of eIPSCs across the stimulus intensities in the group exposed to 1.5 % Iso (red bar, *n*=10 neurons) versus the sham group (black bar, *n*=23 neurons; * indicates *p*<0.05 and ** indicates *p*<0.01 by two-way ANOVA followed with Tukey multiple comparisons).

In summary, our data indicate that single exposure to anesthetic concentrations of Iso alone or to sub-anesthetic concentrations of Iso in combination with N$_2$O in vivo causes a lasting reduction in the amplitudes of eIPSCs that likely is mediated by both presynaptic and postsynaptic mechanisms

as evidenced by the alterations of PPR and decay τs, respectively.

Discusion

It has been well established that even single exposure to anesthesia causes substantial neurodegenerative changes in the brain [2–6]. However, only recently have lasting alterations in synaptic transmission in animals exposed to GAs during brain development been taken into consideration. For example, our previous studies have shown that exposure of rat pups to GAs (triple combination of 0.75 % Iso, 75 % N_2O, and 9 mg/kg midazolam) at the age of P7 causes persisting changes in their brains, including decreased inhibitory synaptic transmission in the subicular pyramidal neurons as evidenced by decreased decay τs, decreased synaptic strength of eIPSCs and alterations of PPR [18]. In our more recent study, we provided the first description of synaptic and intrinsic plasticity in the nRT after single exposure to clinically relevant anesthetics during brain development [14]. We showed that 6 h of exposure of P7 rat pups to anesthesia with 0.75 % Iso, 75 % N_2O, and 9 mg/kg midazolam induces lasting: (1) decrease in synaptic inhibition as demonstrated by diminished strength of eIPSCs, decreased decay τs of eIPSCs and decreased PPR; (2) increase in glutamate-mediated excitatory synaptic transmission as evidenced by increased strength in both evoked and action potential-independent synaptic currents; (3) up-regulation of T-type calcium currents with associated increase in intrinsic excitability manifested by increased ability to fire tonic action potentials and bursts of action potentials; and (4) increased intensity of pharmacologically induced spike-and-wave discharges (SWDs) in intact thalamocortical circuits as measured by EEG recordings in vivo. The SWD discharges are important since they are a hallmark of absence seizures. Here, we find strikingly similar alteration in eIPSCs in nRT cells from rats exposed to the double anesthetic combination of Iso and N_2O or Iso alone. One possible explanation for the changes is that they represent adaptive responses to neuronal loss induced by GAs. It is reasonable to hypothesize that nRT neurons increase their activity to compensate for neuronal loss induced by GAs in order to maintain balance in the circuitry. Unfortunately, this homeostatic plasticity could contribute to chronic hyperexcitability of affected networks (reviewed for GABA receptors by [19]).

Our choice of age P7 for these experiments was based on our previous study, which established that maximal GA-induced neurodegeneration in the rat thalamus is between ages P5 and P10 [20]. It has been shown that activation of $GABA_A$ receptors in developing the nRT at age P3–P8 causes membrane depolarization due to elevated intracellular $[Cl^-]$ [21]. Since it is well-known that Iso is a potent activator of $GABA_A$ receptors in the central nervous system [22], it is possible that

there is a compensatory decrease in the GABA-mediated eIPSCs to dampen any over-excitation of nRT neurons responding to Iso with GABA-mediated depolarization. Our data indicate that this decrease persists later in life when GABA normally becomes an inhibitory transmitter in the CNS due to reversal of the intracellular $[Cl^-]$ gradient. If this hypothesis is correct, then we would expect that other anesthetic drugs that directly stimulate $GABA_A$ receptors, like propofol, may mimic Iso in vivo by decreasing eIPSCs in nRT cells. In contrast, we expect that anesthetic exposure to drugs that do not affect $GABA_A$ receptors, like ketamine, may cause less prominent or no alterations in eIPSCs in nRT cells. Exposure to Iso later in life (after P8) when the intracellular $[Cl^-]$ gradient is well established may be less deleterious. However, other potential effects of GAs on ion channels cannot be discounted. For example, we found that in subicular pyramidal neurons, alterations of eIPSCs are accompanied by ultrastructural changes in mitochondrial integrity and decreased mitochondrial density in the presynaptic terminals [18]. Since we found similar alterations in eIPSCs in nRT neurons after exposure to GAs, it is possible that similar mechanisms could contribute. However, it is also plausible that GA exposure could have selectively decreased density of inhibitory synapses in nRT cells. Future morphological studies are needed to address these issues and will be an important area of our future investigations.

In our very recent study [14], we used a triple combination of potent volatile anesthetic agents (0.75 % Iso, supplemented with 75 % N_2O and 9 mg/kg midazolam) since these agents often are used in combination in clinical anesthesia in order to provide hypnosis, analgesia, and amnesia, respectively. The data we reported here indicate that similar effects on eIPSCs in nRT neurons also are induced by exposing P7 pups to the double combination of GAs: 0.75 % Iso and 75 %N_2O, as well as to higher dose of Iso (1.5 %) alone, but not to 75 % N_2O alone. Furthermore, when a low toxic dose of Iso (0.75 vol%) was combined with a non-toxic dose of nitrous oxide (N_2O) of 75 vol%, this double cocktail resulted in robust neurodegeneration entailing severe damage to the thalamus and parietal cortex, and also producing moderate to severe damage in many other brain regions [2]. Similar severe neurodegeneration was described with double cocktail (Iso, N_2O) but not with the individual agents in non-human primates [3] and primary cortical cultures [23]. We did not test higher full-anesthetic doses of N_2O alone in these experiments since achieving such concentrations would require hyperbaric conditions and that is not relevant to usual clinical practice.

One question that arises is whether it is possible that alterations of eIPSCs in nRT neurons could alone induce observed chronic hyperexcitabaility of intact thalamocortical networks in animals exposed to GAs at P7 as we observed in our recent study [14]. Both human and animal studies have pointed to a potential role of $GABA_A$ receptor dysfunction in absence

seizures [24, 25]. It is of particular interest for this study that intra-nRT connections mediated by long-lasting eIPSCs are critical for regulating inhibitory output and phasic bursting activity. Thus, during thalamocortical oscillation, GABA$_A$-mediated inhibition of nRT cells may prevent the pathological hypersynchrony of absence epilepsy, although intracortical mechanisms also may contribute [9, 26]. Based on our findings that exposure of young rats to common GAs causes lasting loss of inhibitory function of nRT, we proposed that these animals may display increased propensity for absence seizures. Indeed, our in vivo experiments showing enhancement of SWDs strongly support this idea [14]. Along these lines, very similar alterations of eIPSCs in nRT neurons were reported in a genetic model of absence seizures [27]. Specifically, the authors found that in the strain of genetic absence epilepsy rats from Strasbourg (GAERS), eIPSCs in nRT neurons showed small increase in amplitude (25 %) accompanied by more prominent faster decay τ (40 %) and decreased PPR (45 %) as compared with those from non-epileptic age-matched rats. That group subsequently used a computational modeling approach to suggest that the lasting plasticity of eIPSCs alone is sufficient to lower the threshold for excitability in the nRT and consequently in intact thalamocortical networks [28]. Further comprehensive modeling, preclinical and clinical studies are needed to establish a possible link between anesthetic-induced plasticity of eIPSCs in the nRT neurons and abnormal oscillations in thalamocortical networks collectively termed "thalamocortical dysrhythmias" [29]. These and future studies may provide a rationale for new strategies to prevent abnormalities and/or to normalize function of the nRT, the main inhibitory structure in the thalamus, and thus to provide safer anesthesia.

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