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ORIGINAL ARTICLE

Erythropoietin Reduces Neurodegeneration and Long-Term Memory Deficits Following Sevoflurane Exposure in Neonatal Rats

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Abstract

Exposure to general anesthetics induces neural apoptosis and degeneration in the immature neonatal brain. Erythropoietin (EPO) has been shown to protect neonatal animals against hypoxic-ischemic injury and general anesthesia-induced developmental neurotoxicity. However, preventive strategy caused by EPO against neurotoxicity due to general anesthesia is still uncertain. This study examined the effects of EPO administration on brain cytology and cognitive function in adolescent rats exposed to 3% sevoflurane as neonates. Seven-day-old rats received intraperitoneal saline (EPO 0 U group) or EPO (60, 120, or 600 U) 30 min before exposure to 3% sevoflurane with 21% oxygen for 4 h. The rats only received 21% oxygen without EPO and sevoflurane as the sham group. The Morris water maze task was performed time-dependently among the groups, 3 weeks post-anesthesia exposure. Escape latency and % quadrant in the EPO 600 U group were significantly reduced and increased, respectively, compared with those in the EPO 0 U group 6 weeks post-exposure. In addition, freezing time in response to the conditioned stimulus and the number of NeuN-positive cells in the hippocampal CA1 region were significantly increased in the EPO 120 and 600 U groups than in the EPO 0 U group 6 weeks after exposure. Moreover, the statistical parameter mapping of positive cell density was increased in the EPO-treated rats. These results support the observations that pretreatment with EPO reduced long-term cognitive deficits and neuronal degeneration in cortex and hippocampus induced by sevoflurane exposure with low oxygen concentration in neonatal rats.

Keywords Sevoflurane · Anesthesia · Erythropoietin · Cognitive function · Neonate · Neural toxicity

Introduction

Neural toxicity induced by exposure to general anesthesia in neonatal rodents has been shown to cause neurological impairment in later adulthood (Disma et al. 2016; Jevtovic-Todorovic 2018; Jevtovic-Todorovic et al. 2003; Robert 2010; Sanders et al. 2013; Stratmann 2011; Tsuchimoto et al. 2011; Walters and Paule 2017). These reports have shown that general anesthetics induce extensive neuronal apoptosis and degeneration in the developing brain, resulting in long-term neurocognitive deficits. Despite multiple reports of anesthetic neurotoxicity in animals, the results of clinical epidemiological studies in human patients have been controversial (Davidson et al. 2016; Pinyavat et al. 2016; Rappaport et al. 2015; Sun et al. 2016; Warner et al. 2018; Wilder et al. 2009; Yazar et al. 2016). Based on preclinical and clinical reports, the US Food and Drug Administration (FDA) has warned that "repeated or lengthy use of general anesthetic and sedation drugs during surgeries or procedures in children younger than 3 years or in pregnant women during their third trimester may affect the development of children's brains" (Andropoulos and Greene 2017). Additional research is urgently needed to find a solution for this problem.

Erythropoietin (EPO) was originally recognized as a humoral mediator involved in the maturation and proliferation of erythroid progenitor cells (van der Kooij et al. 2008). EPO is also produced in the developing brain and functions as both a growth factor and neuroprotective agent in the central nervous system (Jantzie et al. 2013; Juul and Pet 2015; Yu et al. 2002). EPO exerts its neuroprotective effects through multiple mechanisms, including anti-apoptotic, anti-inflammatory, neuroregenerative, neurotrophic, and anti-oxidant mechanisms (Juul and Pet 2015; van der Kooij et al. 2008).

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Furthermore, EPO ameliorates neurotoxicity induced by N-methyl-D-aspartate receptor antagonists in the developing rat brain (Dzietko et al. 2004).

Pharmacological preventive strategies for neurodegeneration induced by general anesthesia have been introduced (Disma et al. 2016; Walters and Paule 2017). EPO is likely to be one of the possible protective compounds to prevent or ameliorate anesthesia-induced neurodegeneration (Walters and Paule 2017). However, there were few reports which showed that EPO protected neurodegeneration induced by general anesthesia (Pellegrini et al. 2014; Tsuchimoto et al. 2011). Neurodegeneration induced by sevoflurane 2% for 6 h in newborn rat (Pellegrini et al. 2014) and isoflurane 1% for 6 h in the developing mouse brain (Tsuchimoto et al. 2011) were attenuated by a single dose of EPO administration. Although EPO may be effective for the neurodegeneration induced by general anesthesia, there was no dose-response study of EPO nor study with sevoflurane 3% for 4 h in neonatal rodents. In addition, it was not clear whether EPO would affect the more long-term cognitive function in the developing brain. Therefore, preventive strategies of EPO against neurotoxicity induced by general anesthesia are unknown.

We hypothesize that EPO administration could ameliorate neurodegeneration triggered by anesthetics in the developing brain and improve cognitive function in the long term. This study examined the effects of EPO on rat brain histological changes using persistent normal cells and cognitive function in later life following perinatal sevoflurane exposure.

Methods and Materials

Animals

All animal procedures were approved by the animal research committee of Akita University, Japan (Approval number: a-1-2625). This study was performed in accordance with the National Institutes of Health Guide for the Use of Laboratory Animals. A total of 60 pups, Jcl/Wistar rats (Japan Clea, Inc., Japan, 7 days old) weighing 14 ± 3 g were used. We used 7-day-old rat pups in our study because they are at their peak of synaptogenesis, which is equivalent to the neonatal period in humans. The pups were divided into groups with approximately equal numbers of males and females for all experiments. The number and suffering of animals were minimized. An observer who was unaware of the treatment group performed the neurological and histological measurement.

Anesthetic Exposure

On postnatal day 7 (P7), rat pups were injected intraperitoneally with saline or 0–600 U of recombinant human EPO. After 30 min, the pups were placed in a plastic chamber, exposed to 3% sevoflurane with 21% oxygen for 4 h ($n = 12$ per group), and returned to their mother's cage. The rats were divided into five groups: sham, EPO 0 U, EPO 60 U, EPO 120 U, and EPO 600 U. The rats in the sham group received only 21% oxygen during the study period. The total gas flow was 2 L/min. The oxygen and anesthetic agent fractions were measured using a gas analysis system (GE Healthcare Bio-Sciences, Pittsburgh, PA). During exposure to the anesthetic, the chamber was maintained at 30 ± 1 °C with an infrared heat lamp.

Cognitive Function Evaluation

Morris Water Maze

Spatial memory retention was examined as described previously (Goyagi 2018; Goyagi et al. 2012; Morris 1984). The apparatus consisted of a 120-cm-diameter cylindrical tank filled with warm water (20-cm-depth of water), with a transparent 10-cm-diameter platform placed in a constant position in the center of one of the four quadrants. The platform was set 1 cm below the water level. Three weeks after anesthesia exposure (P27-P29), acquisition trials were carried out four times per day for three successive days. Each trial consisted of two sessions. Rats started swimming from one of two fixed positions. The time taken to reach the hidden platform and the swimming path length were measured using a video image motion analyzer (DVTrack DVT-11; Muromachi Kikai Co. Ltd., Tokyo, Japan). If the rat did not reach the hidden platform within 90 s from the start of the swimming session, it was placed onto the platform for 30 s during an acquisition trial. Six weeks after anesthesia exposure (P47-P49), retention trials were carried out four times per day. Rats failing to find the platform within 90 s were not placed onto the platform in this case. A probe trial was not performed during acquisition trials.

Fear-Conditioning Test

Contextual memory retention was examined using fear conditioning as described previously (Goyagi 2018). The apparatus consisted of a clear rectangular Plexiglas box with a floor of parallel metallic rods for the delivery of electric currents (MK-450RSQ; Muromachi Kikai Co., Ltd., Tokyo, Japan). Five weeks post-anesthesia-exposure (P42), the rats were allowed to freely explore their new environment for 1 min. A 70-dB white noise was then delivered for 30 s, at the end of which a concomitant 0.4 mA electric current was delivered through the metallic rods for 1 s. The tone-shock pairing was repeated once a minute for the next 2 min. The animals were left in the cage for an additional 60 s before being returned to their home cage. At 6 weeks post-anesthesia exposure (P49), the rats were placed in an unrelated environment consisting of a triangular arena, the floor and side walls of which were

covered with a black plastic board. The behavior of the animals was recorded for 90 s without any tone and for a further 60 s with the tone used for conditioning. Freezing (no movement except for respiration) was quantified using a video image motion analyzer (DVTrack DVT-11; Muromachi Kikai Co. Ltd., Tokyo, Japan).

Histological Analyses

Neuronal Nuclei Staining

After measuring the freezing and retention times at 6 weeks post-sevoflurane exposure (P49), the rats were anesthetized with 4% halothane in an acrylic plastic box. A high dose of pentobarbital (80 mg/kg) was then administered via intraperitoneal injection, followed by the administration of a direct left ventricular bolus of 0.2 mL heparin. The rat was then transcardially perfused with 100 mL of heparinized saline followed by 150 mL of 4% paraformaldehyde in phosphate buffer (pH 7.4). The brain was removed and embedded in paraffin, and 3-μm-thick serial transverse sections were prepared. After paraffin removal with xylene, the sections were rehydrated, placed in a plastic container filled with citrate buffer (pH 6.0), and heated in a pressure cooker for 10 min at maximum pressure at 121 °C. Endogenous peroxidase activity was quenched with 3% H_2O_2 for 10 min, and the sections were incubated with a mouse monoclonal antibody to NeuN (1:100 dilution; Millipore Corporation, Temecula, CA) for 10 min at 37 °C. Immunodetection was performed using avidin-horse radish peroxidase complexes with biotinylated antibodies to rabbit and mouse IgG (MILLIPORE IHC Select^R Immunoperoxidase Secondary Detection System; Millipore Corporation), with diaminobenzidine as the substrate. The slides were counterstained with hematoxylin. The NeuN-positive cells indicate that the mature typical neurons have existed after growth. The number of NeuN-positive cells in a 500 μm × 300 μm area was counted bilaterally in the CA1 hippocampus and cerebral cortical layer 3 as described previously (Goyagi 2018).

Positive Cell Density Map

The positive cell density map (PCDM) methodology has been described previously (Goyagi 2018; Wada et al. 2012; Wada et al. 2006). In brief, the composite image was FFT-bandpass-filtered with the ImageJ program (National Institute of Health, Bethesda, MD) to eliminate low-frequency drifts (> 20 pixels [50 μm]) and high-frequency noises (< 1 pixel [2.5 μm]). The filtered image was further analyzed with a custom-made program developed in MATLAB with the image processing toolbox (Mathworks Inc., Natick, MA). A NeuN PCDM was prepared for each section by automatic detection and counting of NeuN-positive cells in each 100 μm × 100 μm square compartment. For each rat, we created a PCDM at bregma − 2.5 ± 0.2 mm. Finally, the PCDMs were normalized to a standard section and averaged for each group (Fig. 6a).

Statistical Parameter Mapping of Positive Cell Density

As in previous studies (Goyagi 2018; Wada et al. 2012), we carried out a block-by-block between-group comparison of NeuN-positive cell densities. *T* tests were repeatedly applied to each block, after spatially smoothing each PCDM using a Gaussian filter of the block size (standard deviation [SD] = 100 μm). In blocks where the p value was < 0.05 (uncorrected), we examined whether the EPO 0 U group showed an increased NeuN cell density compared to the EPO-treated groups. The p values from the t tests of each block (< 0.05, uncorrected) were mapped to show areas that might contribute to temporal order judgments (Fig. 6b).

Statistical Analysis

The numbers of NeuN-positive cells, escape latency, swimming time and length, and freezing time are expressed as mean ± SD. Comparisons of these variables among the groups were performed using a one-way or two-way analysis of variance (ANOVA) for multiple comparisons followed by Bonferroni post hoc tests. Differences with p values < 0.05 were considered statistically significant. We performed all analyses using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA).

Results

High-Dose Erythropoietin Improves Memory Retention in Morris Water Maze

The escape latencies and swimming path lengths to the hidden platform in the acquisition trials during P27–29 are shown in Fig. 1. On day 2, the escape latency time in the sham group was significantly less than that in the EPO 60 U group. The swimming path length in the sham group was significantly shorter than the other EPO-reated groups on day 1. On day 2, the path lengths in the sham group were significantly shorter than the EPO 0, 60, and 600 U group; on day 3, the path lengths in the sham group were significantly shorter than the EPO 0 and 60 U groups. The swimming speed of the sham group on day 1 was significantly slower compared with the EPO 60 and 600 U groups, and there were significant differences between the EPO 0 U and 60 U, and between the EPO 0 U and 600 U groups, respectively. On day 2, the swimming speed of the sham group was significantly faster than the EPO 0, 120, and 600 U groups. On day 3, the speed of the sham group was significantly faster compared with the other groups. Acquired learning significantly improved time-dependently.

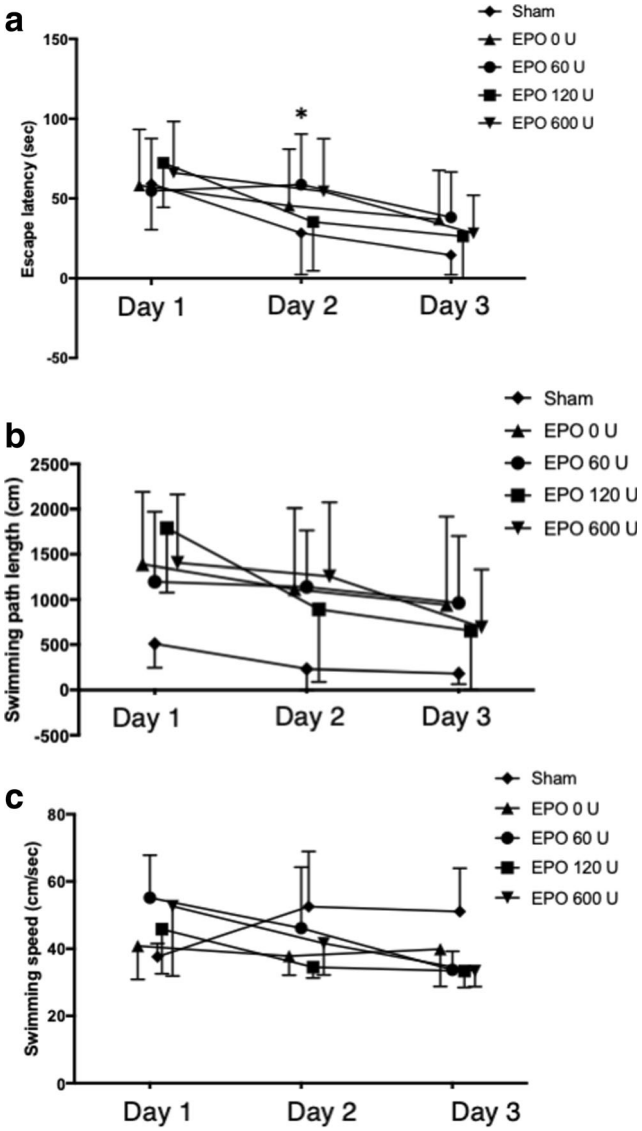

Fig. 1 Morris water maze results 3 weeks after sevoflurane exposure. **a** Escape latency to reach the platform. **b** Swimming path length to the platform. **c** Swimming speed were measured. Days 1, 2, and 3 of the acquisition trials were P27, P28, and P29, respectively. The escape latency time in the sham group was significantly decreased compared with that in the EPO 60 U group on day 2. The swimming path length in the sham group was significantly shorter than the other EPO-treated groups on day 1. On day 2, the length in the sham group was significantly shorter than the EPO 0, 60, and 600 U groups, and on day 3, the length in the sham group was significantly shorter than the EPO 0 U and 60 U groups. The swimming speed in the sham group on day 1 was significantly decreased compared with that in the EPO 60 U and 600 U groups, and there were significant differences between the EPO 0 U and 60 U and between the EPO 0 U and 600 U group, respectively. The speed in the sham group on day 2 was significantly increased compared with that in the EPO 0, 120, and 600 U groups. On day 3, the speed in the sham group was significantly increased compared with that in the other groups. Acquired learning significantly improved time-dependently. The data are represented as means ± SDs ($n = 12$ per group). EPO, erythropoietin

As shown in Fig. 2, the escape latencies in the EPO 0 U group were significantly longer than those in the sham and EPO 600 U groups at P47, whereas no significant differences were observed in the swimming path length among the groups. Percent quadrant in the EPO 0 and 60 U group significantly decreased compared with the sham group. There was no difference in the % quadrant between the sham and EPO 600 U groups, which suggested EPO 600 U improved it.

Erythropoietin Improves Anesthetic-Induced Memory Impairment of Cued Fear-Conditioning Test

The freezing times in response to the conditioned stimulus tone in the EPO 0 and 60 U group significantly decreased compared with the sham group, whereas the times in the EPO-treated groups were longer than that in the EPO 0 U group, suggesting that the administration of EPO improved memory impairment in adolescent rats exposed to anesthesia in the developing brain (Fig. 3).

Erythropoietin Attenuates the Reduction in Neuron Numbers in Anesthesia-Exposed Brains

Figure 4 shows NeuN staining of brain sections from the 5 groups at − 2.5 mm caudally to bregma. The number of NeuN-positive cells per 0.15 mm^2 in the hippocampal CA1 region significantly increased in the EPO 120 and 600 U group compared with the EPO 0 U group (Fig. 5a). In addition, the number of NeuN-positive cells in the cortex significantly increased in the EPO 600 U and sham group compared with the EPO 0 U group (Fig. 5b).

Positive Cell Density Map and Statistical Parameter Mapping of Positive Cell Density

Figure 6a shows the NeuN PCDM for each group, which tended to increase in the cortex. EPO-treated rats showed an increase in the hippocampal and cortical PCDM compared with the EPO 0 U group. The differences among the groups are shown in more detail in Fig. 6b. EPO-treated rats (EPO 60 U, 120 U, and 600 U groups) had significantly increased NeuN-positive cell densities when compared to that in the EPO 0 U group. Increased NeuN expression was observed in almost all cortical and hippocampal areas, indicating that the numbers of normal cells in the EPO-treated groups were higher than in the EPO 0 U group.

Discussion

This study shows that EPO preconditioning before exposure to sevoflurane in infant rats improves long-term performance in the water maze task and fear conditioning test during adolescence and increases the number of mature neurons in the CA1 hippocampus and cortex, compared with the previous

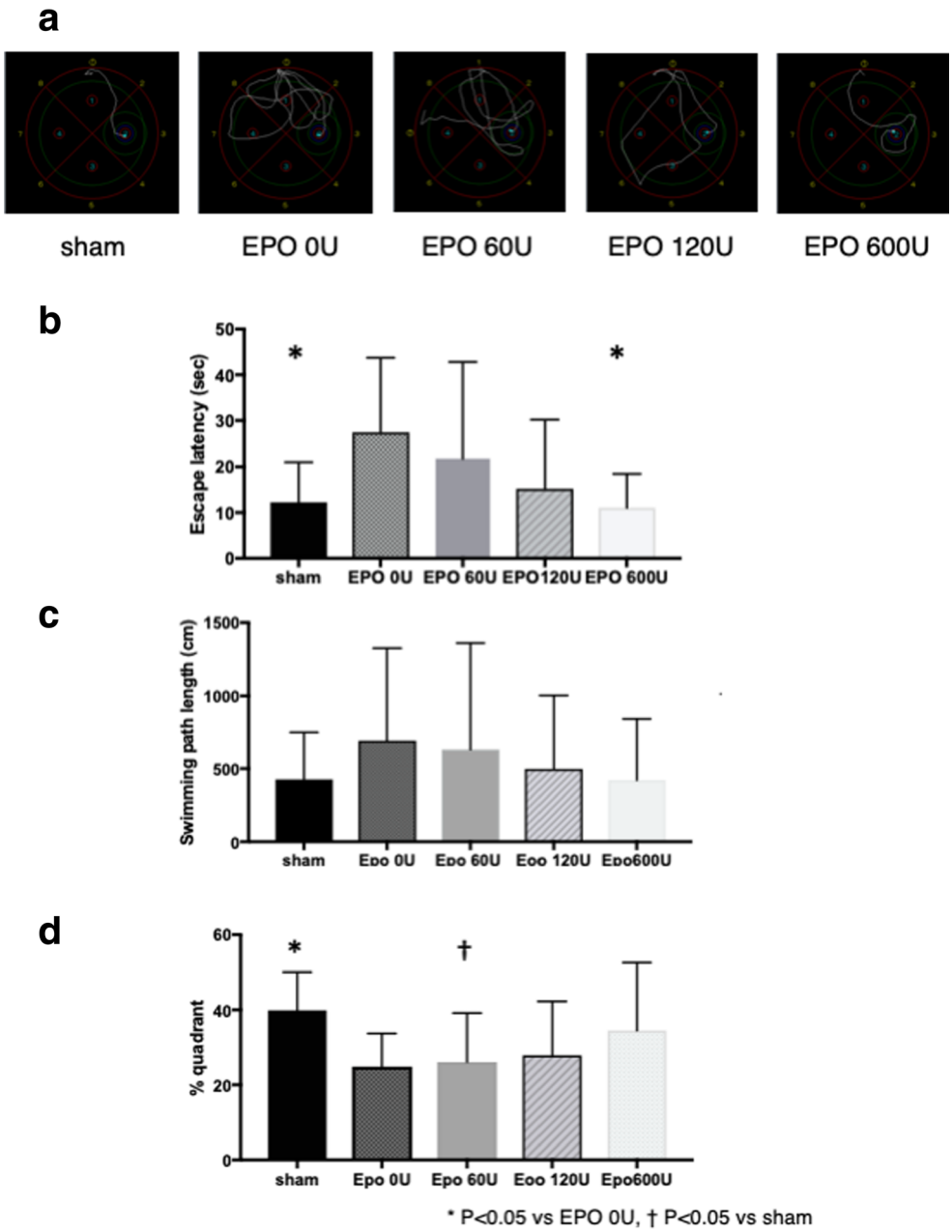

Fig. 2 Morris water maze results 6 weeks after sevoflurane exposure. **a** Typical route maps of Morris water maze at P47, **b** escape latency to reach the platform, **c** swimming path length, and **d** % quadrant were measured on day 1 (P47). The escape latency in the EPO 0 U group was significantly longer than that in the sham and EPO 600 U group at P47, whereas no significant differences were observed in the swimming path length among the groups. Percent quadrant in the EPO 0 and 60 U groups was significantly decreased compared with that in the sham group. There was no difference of % quadrant between the sham and EPO 600 U group. *$P < 0.05$ vs. EPO 0 U. The data are represented as means ± SDs ($n = 12$ per group). EPO, erythropoietin

studies. These results suggest that pretreatment with EPO can ameliorate sevoflurane-induced neural toxicity in the neonatal brain.

Neural toxicity induced by exposure to general anesthesia in neonatal rodents has been shown to lead to neurological impairment in adulthood (Disma et al. 2016; Jevtovic-Todorovic et al. 2003; Robert 2010; Sanders et al. 2013; Stratmann 2011; Walters and Paule 2017). Although numerous animal studies have reported anesthetic neurotoxicity, the results of clinical epidemiological studies in human patients have been controversial (Andropoulos and Greene 2017; Davidson et al. 2016; Jevtovic-Todorovic 2018; Pinyavat et al. 2016; Rappaport et al. 2015; Sun et al. 2016; Warner et al. 2018; Yazar et al. 2016). We have previously reported that sevoflurane-induced neurodegeneration is reduced by high oxygen concentrations as a carrier gas (Goyagi 2018). Therefore, we used 21% oxygen as a carrier for sevoflurane anesthesia to minimize the neuroprotective effect of oxygen.

EPO is a 30.4-kDa cytokine that was originally identified based on its role in erythropoiesis. EPO is produced primarily in the kidney after birth. EPO is also produced in the developing brain, where it acts as both a growth factor and neuroprotective agent (Jantzie et al. 2013; Juul and Pet 2015; Yu et al. 2002). The neuroprotective effects of EPO are mediated by multiple mechanisms (Juul and Pet 2015; van der Kooij et al. 2008). Both in vivo and in vitro studies have shown that

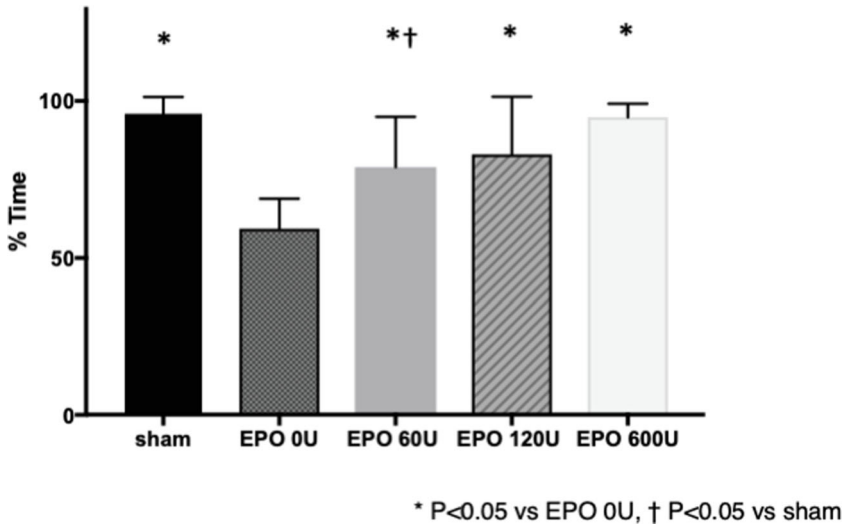

Fig. 3 Fear-conditioning test results. Time spent freezing in response to the conditioned stimulus tone in the EPO 0 and 60 U groups was significantly decreased compared with that in the sham group, whereas the times in the EPO-treated groups were longer than that in the EPO 0 U group. *$P < 0.05$ vs. EPO 0 U. The data are represented as means ± SDs ($n = 12$ per group). EPO, erythropoietin

EPO can reduce hypoxic-ischemic brain injury in animals (Digicaylioglu and Lipton 2001; Iwai et al. 2010; Jantzie et al. 2013; Juul and Pet 2015; Kumral et al. 2004; McPherson and Juul 2010; Sinor and Greenberg 2000) and humans (Elmahdy et al. 2010; Rogers et al. 2014; Wu et al. 2012; Zhu et al. 2009). Moreover, EPO attenuates inhalational-anesthesia-induced neurodegeneration and learning deficits in the developing rodent brain (Pellegrini et al. 2014; Tsuchimoto et al. 2011). EPO is likely not to across the blood brain barrier (BBB) because of its large molecule. Since BBB seems to be easily disrupted after the insults such as anesthetic exposure (Acharya et al. 2015), some EPO can cross the BBB during and after the anesthesia exposure. Therefore, EPO is likely to act both centrally and peripherally.

The results of this dose-response study are consistent with those of two previous reports. First, Pellegrini et al. demonstrated that intraperitoneal recombinant human EPO (rhEPO; 5000 IU/kg) reduced both early activation of apoptosis and late-onset neurologic disorders (as assessed by the water maze and novel object tests) induced by 6-h 2% sevoflurane anesthesia in neonatal rats (Pellegrini et al. 2014). The authors also demonstrated that rhEPO stimulated the expression of nerve growth factor (NGF) and brain-derived growth factor (BDNF) after sevoflurane exposure. Second, Tsuchimoto et al. showed

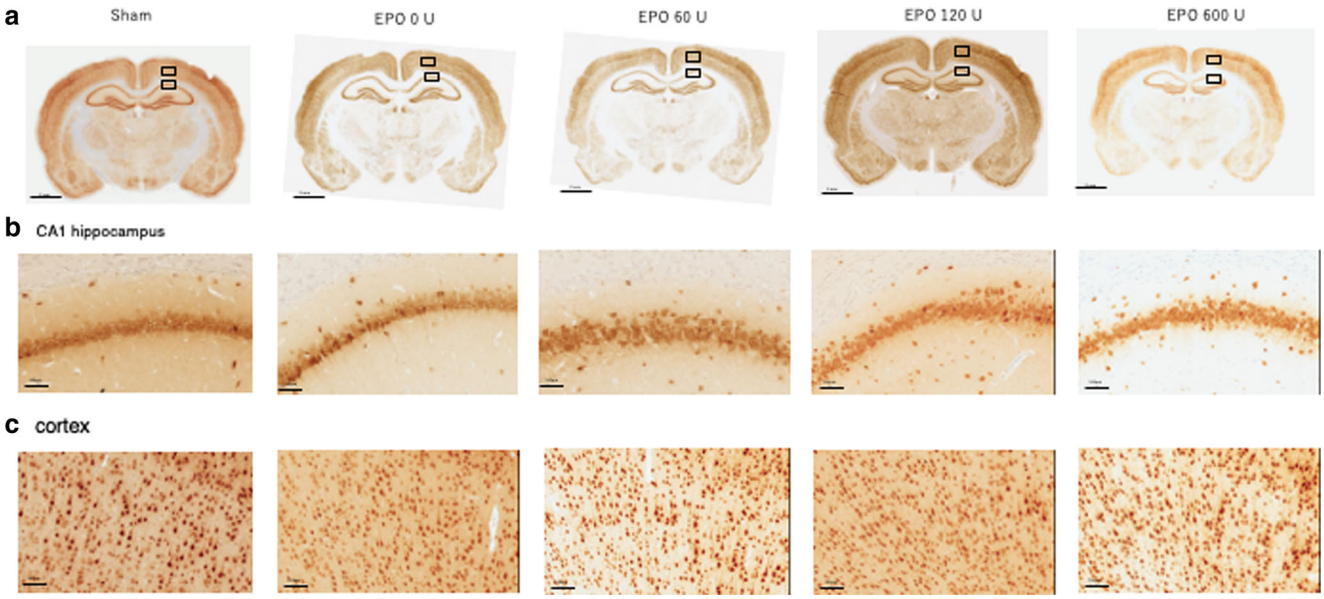

Fig. 4 NeuN staining of brain sections from sevoflurane-exposed rats. Representative NeuN staining at − 2.5 ± 0.2 mm from bregma is shown. NeuN-positive cells in the entire image at lower magnification (**a**), CA1 hippocampus at higher magnification (**b**), and cortex at higher magnification (**c**) are shown for each group. Squares indicate the measuring field in the cortex and CA1. Scale bar: 2 mm (**a**) or 100 μm (**b**, **c**). EPO, erythropoietin

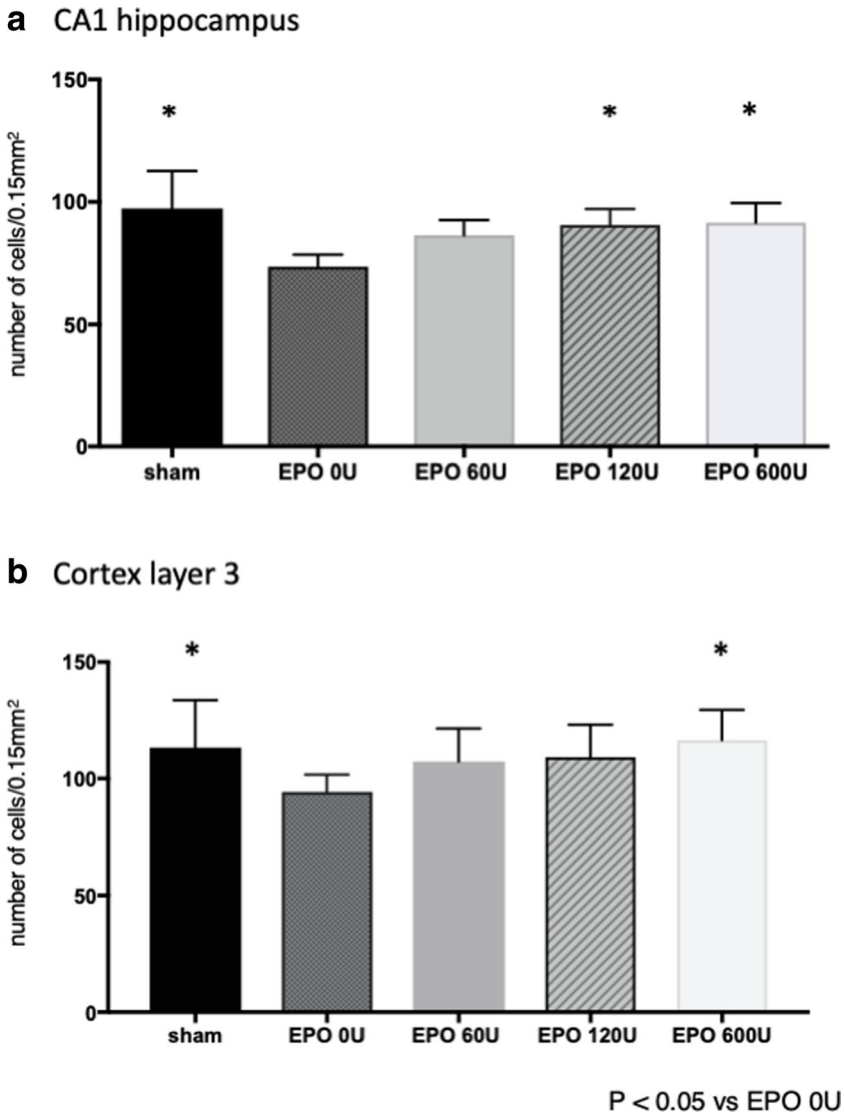

Fig. 5 Numbers of NeuN-positive cells in the cortex and hippocampus in sevoflurane-exposed rats. The number of NeuN-positive cells in the CA1 region of the hippocampus was significantly increased in the EPO 120 and 600 U group compared with that in the EPO 0 U group (**a**). The number of positive cells in the cortex was significantly increased in the EPO 600 U and sham groups compared with that in the EPO 0 U group (**b**). *$P < 0.05$ vs EPO 0 U. The data are represented as means ± SDs ($n = 12$ per group). EPO, erythropoietin

that subcutaneous injection of 50,000 IU/kg rhEPO improved spatial memory and reduced histological alterations in CA1–3 and dentate neurons induced by 6-h 1% isoflurane exposure in neonatal mice (Tsuchimoto et al. 2011). We used 60 U (4285.7 U/kg), 120 U (8,571 U/kg), and 600 U (42,857 U/kg) of rhEPO. Although 60 U of EPO may appear as a small dose compared to those used in the previous reports, we found it efficacious in preserving cognitive function as evaluated by the fear conditioning test and in maintaining the normal-cell count in the cortex and hippocampus. In addition, we found an increased PCDM in EPO-treated rats using Wada's method (Wada et al. 2012; Wada et al. 2006). Although the escape latency in the EPO 600 U group significantly decreased compared with the EPO 0 U group, the swimming path length in the EPO 600 U group tended to be shorter without being statistically significant. Even though we found a significant increase in freezing time and normal-cell count in all EPO-treated groups, the escape latencies at P47 were significantly shorter only in the EPO 600 U group. These results suggest that the minimum dose of EPO required to reduce anesthetic toxicity effectively is likely to be 600 U (42,857 U/kg). Although we did not measure probe trials accurately during the retention trial in the Morris water maze task, % quadrant in the EPO 0 and 60 U group significantly decreased compared with that in the sham group.

The mechanisms of EPO-induced neuroprotection were not addressed in the present study. However, one may speculate that anti-apoptotic, anti-inflammatory, neuroregenerative, and anti-oxidant effects were involved, as described in the previous reports (Juul and Pet 2015; van der Kooij et al. 2008). Thus, Pellegrini et al. reported that rhEPO reduced the number of apoptotic cells on day 3 after sevoflurane exposure (Pellegrini et al. 2014). In addition, rhEPO increased BDNF and NGF expression (Pellegrini et al. 2014). Further study is needed to clarify the mechanisms of EPO-induced neuroprotection against anesthetic neurotoxicity in the

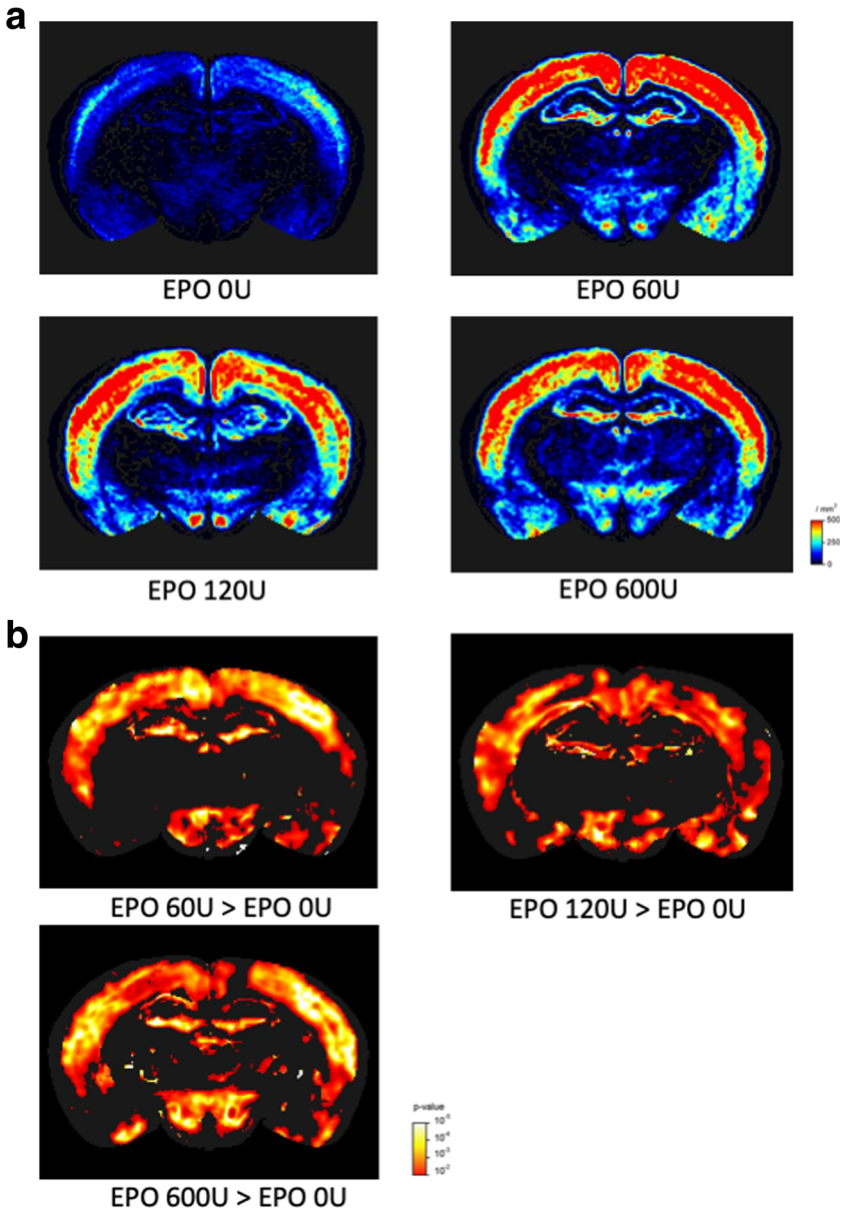

Fig. 6 Positive cell density map (PCDM) and statistical parameter mapping of positive cell densities in the brains of sevoflurane-exposed rats. **a** NeuN PCDM for each group is shown. **b** Statistical parametric mapping showing the areas (red) where the EPO-treated group yielded significantly higher NeuN-positive cell densities than those in the EPO 0 U group. The numbers of normal cells in the EPO-treated group were higher than in the EPO 0 U group both in the cortex and hippocampus. EPO, erythropoietin

developing brain. The clinical relevance of the findings of the present study remains unclear. Since the clinical trials of EPO for hypoxic-ischemic encephalopathy are ongoing, the effects of EPO on anesthetic toxicity in the developing human brain will be considered beneficial.

There are several limitations in this study. First, we used air as the carrier gas during the sevoflurane exposure in this study. EPO has a neuroprotective effect against hypoxic-ischemic injury in the neonatal brain (Jantzie et al. 2013; Juul and Pet 2015; Olgun et al. 2013; Yu et al. 2002; Zhu et al. 2014). Although the hypoxic-ischemic encephalopathy was induced by occlusion of artery or 6–8% oxygen supply in previous animal models (Juul and Pet 2015; Olgun et al. 2013; Zhu et al. 2014), resulted in extremely low PaO_2 that were likely to be very lower than that in this study, there is a possibility that EPO might be protecting from the low arterial oxygen level induced by 21% oxygen rather than neurotoxicity induced by sevoflurane in this study. According to our previous study, the values of PaO_2 were not significantly different between the 21% and 30% oxygen group under sevoflurane anesthesia, but these values significantly decreased compared with those in the control group (that means sham group in this study) (Goyagi 2018). The pups that received sevoflurane with 21% oxygen exhibited a profound increase in neurodegeneration compared with those receiving sevoflurane with 30% oxygen (Goyagi 2018). Although a carrier gas with 21% oxygen compared with higher oxygen concentrations might reinforce neurodegeneration induced by sevoflurane anesthesia in the neonatal brain, EPO ameliorated the neurodegeneration induced by sevoflurane exposure with 21%

oxygen in this study. Moreover, the pups in the sham group that received 21% oxygen alone as a carrier gas without EPO did not exhibit neurodegeneration. As a result, the reduction in neurodegeneration due to EPO administration may be due not only to the effect of EPO on low arterial blood oxygen levels but also to the improved effect on neurotoxicity induced by both sevoflurane and low arterial oxygen levels. Second, the randomization in this study was not strict. Since two pups in the group were selected from their dam, distribution of the pups from their dams were equal. In addition, in all experiments, the groups had approximately equal numbers of males and females. Although confounding factors related to gender are negligible, genetic factors among the groups might affect the result of this study because of the inappropriate randomization. Third, though we used the positive cell density map to compare the NeuN-positive cells similar to previous studies (Goyagi 2018; Wada et al. 2006), this method seems not to be a gold standard. However, the difference in positive cells seems to be easily understood for colored areas, regardless of the contrast difference of each slide (Wada et al. 2006). Fourth, EPO was administered once in this study. Although the effect of EPO on the neuronal degeneration induced by sevoflurane anesthesia was similar to that in previous studies, further studies are needed to establish a clear suitable dose and timing of administration. Further research is necessary to clarify the effects of EPO on other cognitive tasks.

As we mentioned above, the values of PaO_2 were not significantly different between the 21% and 30% oxygen group, but these values significantly decreased compared with those in the no sevoflurane group (Goyagi 2018). One of the reasons is that sevoflurane can cause respiratory depression. In clinical situations, children are usually anesthetized with oxygen to avoid hypoxia. Therefore, the result of this experiment simply does not match the clinical situation. Nonetheless, it is valuable and important for pediatric anesthesia to show that EPO exerts a neuroprotective effect on sevoflurane-induced neurotoxicity. Consequently, we investigated the effect of EPO on sevoflurane-induced neurotoxicity using rats under strict situation. Further studies are needed to make EPO available for pediatric anesthesia to reduce neurotoxicity.

In conclusion, 4-h administration of sevoflurane anesthesia with low oxygen concentration in neonatal rats resulted in significant cognitive impairment in adulthood. A single administration of EPO (600 U) before sevoflurane exposure reduced the neurologic impairment. In addition, even a small dose of EPO increased the number of intact neurons in the hippocampus exposed to sevoflurane as developing brain. The increase in neuron numbers was associated with improved cognitive function. EPO may hold promise as a neuroprotective agent in children's anesthesia.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

Ethical Approval The animal experiments were performed according to international ethical standards and approved by the research ethics committee of Akita University (a-1-2625). Every effort was made to minimize the number and suffering of animals.

References

Acharya NK, Goldwaser EL, Forsberg MM, Godsey GA, Johnson CA, Sarkar A, DeMarshall C, Kosciuk MC, Dash JM, Hale CP, Leonard DM, Appelt DM, Nagele RG (2015) Sevoflurane and isoflurane induce structural changes in brain vascular endothelial cells and increase blood-brain barrier permeability: possible link to postoperative delirium and cognitive decline. Brain Res 1620:29–41. https://doi.org/10.1016/j.brainres.2015.04.054

Andropoulos DB, Greene MF (2017) Anesthesia and developing brains—implications of the FDA warning. N Engl J Med 376: 905–907

Davidson AJ, Disma N, de Graaff JC, Withington DE, Dorris L, Bell G, Stargatt R, Bellinger DC, Schuster T, Arnup SJ, Hardy P, Hunt RW, Takagi MJ, Giribaldi G, Hartmann PL, Salvo I, Morton NS, von Ungern Sternberg B, Locatelli BG, Wilton N, Lynn A, Thomas JJ, Polaner D, Bagshaw O, Szmuk P, Absalom AR, Frawley G, Berde C, Ormond GD, Marmor J, McCann M, GAS consortium (2016) Neurodevelopmental outcome at 2 years of age after general anaesthesia and awake-regional anaesthesia in infancy (GAS): an international multicentre, randomised controlled trial. Lancet 387:239–250. https://doi.org/10.1016/s0140-6736(15)00608-x

Digicaylioglu M, Lipton SA (2001) Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-κB signalling cascades. Nature 412:641–647

Disma N, Mondardini MC, Terrando N, Absalom AR, Bilotta F (2016) A systematic review of methodology applied during preclinical anesthetic neurotoxicity studies: important issues and lessons relevant to the design of future clinical research. Paediatr Anaesth 26:6–36. https://doi.org/10.1111/pan.12786

Dzietko M, Felderhoff-Mueser U, Sifringer M, Krutz B, Bittigau P, Thor F, Heumann R, Bührer C, Ikonomidou C, Hansen HH (2004) Erythropoietin protects the developing brain against N-methyl-D-aspartate receptor antagonist neurotoxicity. Neurobiol Dis 15:177–187. https://doi.org/10.1016/j.nbd.2003.10.006

Elmahdy H, El-Mashad AR, El-Bahrawy H, El-Gohary T, El-Barbary A et al (2010) Human recombinant erythropoietin in asphyxia neonatorum: pilot trial. Pediatrics 125:e1135–e1142. https://doi.org/10.1542/peds.2009-2268

Goyagi T (2018) The additional oxygen as a carrier gas during long-duration sevoflurane exposure ameliorate the neuronal apoptosis

and improve the long-term cognitive function in neonatal rats. Brain Res 1678:220–230. https://doi.org/10.1016/j.brainres.2017.10.014

Goyagi T, Tobe Y, Nishikawa T (2012) Long-term and spatial memory effects of selective beta1-antagonists after transient focal ischaemia in rats. Br J Anaesth 109:399–406. https://doi.org/10.1093/bja/aes134

Iwai M, Stetler RA, Xing J, Hu X, Gao Y, Zhang W, Chen J, Cao G (2010) Enhanced oligodendrogenesis and recovery of neurological function by erythropoietin after neonatal hypoxic/ischemic brain injury. Stroke 41:1032–1037. https://doi.org/10.1161/STROKEAHA.109.570325

Jantzie LL, Miller RH, Robinson S (2013) Erythropoietin signaling promotes oligodendrocyte development following prenatal systemic hypoxic-ischemic brain injury. Pediatr Res 74:658–667. https://doi.org/10.1038/pr.2013.155

Jevtovic-Todorovic V (2018) Exposure of developing brain to general anesthesia: what is the animal evidence? Anesthesiology 128:832–839. https://doi.org/10.1097/ALN.0000000000002047

Jevtovic-Todorovic V, Hartman ER, Izumi Y, Benshoff DN, Dikranian K et al (2003) Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. J Neurosci 23:876–882

Juul SE, Pet GC (2015) Erythropoietin and neonatal neuroprotection. Clin Perinatol 42:469–481. https://doi.org/10.1016/j.clp.2015.04.004

Kumral A, Uysal N, Tugyan K, Sonmez A, Yilmaz O, Gokmen N, Kiray M, Genc S, Duman N, Koroglu TF, Ozkan H, Genc K (2004) Erythropoietin improves long-term spatial memory deficits and brain injury following neonatal hypoxia-ischemia in rats. Behav Brain Res 153:77–86. https://doi.org/10.1016/j.bbr.2003.11.002

McPherson RJ, Juul SE (2010) Erythropoietin for infants with hypoxic-ischemic encephalopathy. Curr Opin Pediatr 22:139–145. https://doi.org/10.1097/MOP.0b013e328336eb57

Morris R (1984) Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 11:47–60

Olgun Y, Kirkim G, Kolatan E, Kiray M, Bagriyanik A et al (2013) Otoprotective effect of recombinant erythropoietin in a model of newborn hypoxic-ischemic encephalopathy. Int J Pediatr Otorhinolaryngol 77:739–746. https://doi.org/10.1016/j.ijporl.2013.01.029

Pellegrini L, Bennis Y, Velly L, Grandvuillemin I, Pisano P, Bruder N, Guillet B (2014) Erythropoietin protects newborn rat against sevoflurane-induced neurotoxicity. Paediatr Anaesth 24:749–759. https://doi.org/10.1111/pan.12372

Pinyavat T, Warner DO, Flick RP, McCann ME, Andropoulos DB et al (2016) Summary of the update session on clinical neurotoxicity studies. J Neurosurg Anesthesiol 28:356–360. https://doi.org/10.1097/ANA.0000000000000347

Rappaport BA, Suresh S, Hertz S, Evers AS, Orser BA (2015) Anesthetic neurotoxicity—clinical implications of animal models. N Engl J Med 372:796–797. https://doi.org/10.1056/NEJMp1414786

Robert WT (2010) Is there any relationship between long-term behavior disturbance and early exposure to anesthesia? Curr Opin Anaesthesiol 23:332–336. https://doi.org/10.1097/ACO.0b013e3283391f94

Rogers EE, Bonifacio SL, Glass HC, Juul SE, Chang T, Mayock DE, Durand DJ, Song D, Barkovich AJ, Ballard RA, Wu YW (2014) Erythropoietin and hypothermia for hypoxic-ischemic encephalopathy. Pediatr Neurol 51:657–662. https://doi.org/10.1016/j.pediatrneurol.2014.08.010

Sanders RD, Hassell J, Davidson AJ, Robertson NJ, Ma D (2013) Impact of anaesthetics and surgery on neurodevelopment: an update. Br J Anaesth 110(Suppl 1):i53–i72. https://doi.org/10.1093/bja/aet054

Sinor AD, Greenberg DA (2000) Erythropoietin protects cultured cortical neurons, but not astroglia, from hypoxia and AMPA toxicity. Neurosci Lett 290:213–215

Stratmann G (2011) Neurotoxicity of anesthetic drugs in the developing brain. Anesth Analg 113:1170–1179. https://doi.org/10.1213/ANE.0b013e318232066c

Sun LS, Li G, Miller TL, Salorio C, Byrne MW et al (2016) Association between a single general anesthesia exposure before age 36 months and neurocognitive outcomes in later childhood. JAMA 315:2312–2320. https://doi.org/10.1001/jama.2016.6967

Tsuchimoto T, Ueki M, Miki T, Morishita J, Maekawa N (2011) Erythropoietin attenuates isoflurane-induced neurodegeneration and learning deficits in the developing mouse brain. Paediatr Anaesth 21:1209–1213. https://doi.org/10.1111/j.1460-9592.2011.03657.x

van der Kooij MA, Groenendaal F, Kavelaars A, Heijnen CJ, van Bel F (2008) Neuroprotective properties and mechanisms of erythropoietin in in vitro and in vivo experimental models for hypoxia/ischemia. Brain Res Rev 59:22–33. https://doi.org/10.1016/j.brainresrev.2008.04.007

Wada M, Yoshimi K, Higo N, Ren YR, Mochizuki H, Mizuno Y, Kitazawa S (2006) Statistical parametric mapping of immunopositive cell density. Neurosci Res 56:96–102. https://doi.org/10.1016/j.neures.2006.05.013

Wada M, Takano K, Ikegami S, Ora H, Spence C, Kansaku K (2012) Spatio-temporal updating in the left posterior parietal cortex. PLoS One 7:e39800. https://doi.org/10.1371/journal.pone.0039800

Walters JL, Paule MG (2017) Review of preclinical studies on pediatric general anesthesia-induced developmental neurotoxicity. Neurotoxicol Teratol 60:2–23. https://doi.org/10.1016/j.ntt.2016.11.005

Warner DO, Zaccariello MJ, Katusic SK, Schroeder DR, Hanson AC, Schulte PJ, Buenvenida SL, Gleich SJ, Wilder RT, Sprung J, Hu D, Voigt RG, Paule MG, Chelonis JJ, Flick RP (2018) Neuropsychological and behavioral outcomes after exposure of young children to procedures requiring general anesthesia: the mayo anesthesia safety in kids (MASK) study. Anesthesiology 129:89–105. https://doi.org/10.1097/ALN.0000000000002232

Wilder RT, Flick RP, Sprung J, Katusic SK, Barbaresi WJ, Mickelson C, Gleich SJ, Schroeder DR, Weaver AL, Warner DO (2009) Early exposure to anesthesia and learning disabilities in a population-based birth cohort. Anesthesiology 110:796–804

Wu YW, Bauer LA, Ballard RA, Ferriero DM, Glidden DV, Mayock DE, Chang T, Durand DJ, Song D, Bonifacio SL, Gonzalez FF, Glass HC, Juul SE (2012) Erythropoietin for neuroprotection in neonatal encephalopathy: safety and pharmacokinetics. Pediatrics 130:683–691. https://doi.org/10.1542/peds.2012-0498

Yazar S, Hewitt AW, Forward H, Jacques A, Ing C, von Ungern-Sternberg BS, Mackey DA (2016) Early anesthesia exposure and the effect on visual acuity, refractive error, and retinal nerve fiber layer thickness of young adults. J Pediatr 169:256–259. https://doi.org/10.1016/j.jpeds.2015.10.048

Yu X, Shacka JJ, Eells JB, Suarez-Quian C, Przygodzki RM, Beleslin-Cokic B, Lin CS, Nikodem VM, Hempstead B, Flanders KC, Costantini F, Noguchi CT (2002) Erythropoietin receptor signalling is required for normal brain development. Development 129:505–516

Zhu C, Kang W, Xu F, Cheng X, Zhang Z, Jia L, Ji L, Guo X, Xiong H, Simbruner G, Blomgren K, Wang X (2009) Erythropoietin improved neurologic outcomes in newborns with hypoxic-ischemic encephalopathy. Pediatrics 124:e218–e226. https://doi.org/10.1542/peds.2008-3553

Zhu L, Bai X, Wang S, Hu Y, Wang T, Qian L, Jiang L (2014) Recombinant human erythropoietin augments angiogenic responses in a neonatal rat model of cerebral unilateral hypoxia-ischemia. Neonatology 106:143–148. https://doi.org/10.1159/000362262