Long-term Effects of Single or Multiple Neonatal Sevoflurane Exposures on Rat Hippocampal Ultrastructure

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ABSTRACT

Background: Neonatal exposure to general anesthetics may pose significant neurocognitive risk. Human epidemiological studies demonstrate higher rates of learning disability among children with multiple, but not single, exposures to anesthesia. The authors employ a rat model to provide a histological correlate for these population-based observations. The authors examined long-term differences in hippocampal synaptic density, mitochondrial density, and dendritic spine morphology.

Methods: Twenty male rat pups (n = 5/condition) were exposed to 2.5% sevoflurane under one of four conditions: single 2-h exposure on postnatal day 7 (P7); single 6-h exposure on P7; repeated 2-h exposures on P7, P10, and P13 for a cumulative 6 h of general anesthetics; or control exposure to 30% oxygen on P7, P10, and P13.

Results: Repeated exposure to general anesthetics resulted in greater synaptic loss relative to a single 2-h exposure ($P < 0.001$). The magnitude of synaptic loss induced by three 2-h exposures ($1.977 \pm 0.040\ \mu m^3$ [mean ± SEM]) was more profound than that of a single 6-h exposure ($2.280 \pm 0.045\ \mu m^3$, $P = 0.022$). Repeated exposures did not alter the distribution of postsynaptic density length, indicating a uniform pattern of loss across spine types. In contrast, mitochondrial toxicity was best predicted by the cumulative duration of exposure. Relative to control (0.595 ± 0.017), both repeated 2-h exposures (0.479 ± 0.015) and a single 6-h exposure (0.488 ± 0.013) were associated with equivalent reductions in the fraction of presynaptic terminals containing mitochondria ($P < 0.001$).

Conclusion: This suggests a "threshold effect" for general anesthetic–induced neurotoxicity, whereby even brief exposures induce long-lasting alterations in neuronal circuitry and sensitize surviving synapses to subsequent loss. **(ANESTHESIOLOGY 2015; 122:87-95)**

OVER the past 30 yr advances in medical and surgical techniques have proven lifesaving for young children. As a consequence, early and repeated exposure to general anesthetics (GA) has increased. Yet recent evidence suggests that such practices pose significant neurocognitive risk. Human epidemiological studies demonstrate that childhood exposure to GA is associated with an increased risk for the later development of cognitive and behavioral impairment.[1,2] This risk is especially high among children with multiple anesthetic exposures.[3–5] However, the clinical implications and mechanism underlying these findings remain unclear. These studies are retrospective in design and therefore limited in their ability to distinguish between the effects of anesthetic-induced neurotoxicity and confounders such as coexistent medical disease, surgical stress, and hospitalization.

In vivo animal studies demonstrate that GA is immediately toxic to the developing brain, and even a single early exposure induces long-term cognitive and behavioral deficits.[6–8] These deficits have been linked to disturbances in neuronal circuitry, mitochondrial morphology, and dendritic spine development.[9–12] Our previous work demonstrates that relative to a single neonatal exposure, rats repeatedly exposed to

What We Already Know about This Topic

- Human epidemiological studies suggest that children exposed to multipleanesthetics, as opposed to a single anesthetic, might be at greater risk for development of cognitive dysfunction
- The underlying mechanism by which injury is increased with multiple exposures is not known
- The effect of a single exposure or multiple exposures with equivalent total duration of exposure on brain ultrastructure was evaluated by electron microscopy in rodents

What This Article Tells Us That Is New

- Repeated exposure to sevoflurane led to a greater loss of synapses in comparison to a single exposure
- Anesthetic exposure led to a reduction in the number of synaptic terminals with mitochondria
- Interestingly, this reduction was correlated to total anesthetic exposure rather than frequency of exposure
- These data suggest that a brief anesthetic exposure might sensitize the brain to subsequent anesthetic induced injury

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isoflurane had greater deficits in spatial working memory.[13] We therefore hypothesize that, as compared to a single exposure, brief but repeated neonatal exposures would produce greater neurohistologic damage.

Characterizing the dose-related effects of neonatal anesthetic exposure is critical for the development of safe clinical practices and informed preoperative counseling. Here, we use a rat model to investigate the long-term ultrastructural effects of single or multiple neonatal exposures to GA. Specifically, we examine differences in synaptic density, mitochondrial density, and dendritic spine head morphology. Our study demonstrates that relative to a single exposure, repeated neonatal exposure to GA is highly neurotoxic. Although alterations in presynaptic mitochondrial localization appear to be dependent on the cumulative duration of exposure, synaptic losses are best predicted by the total number of anesthetic exposures.

Materials and Methods

Animals

All experiments were carried out in strict accordance with the recommendations of the Guide for the Care and of Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD). The protocol (LA10-00071) was approved by the Icahn School of Medicine Animal Use and Care Committee (New York, New York).

Male Long-Evans rat pups from six natural litters were randomly divided into four balanced experimental groups. Rats were then exposed to 2.5% sevoflurane in 30% oxygen under one of four conditions: (1 × 2 h) a single 2-h exposure on postnatal day 7 (P7), followed by control exposure to 30% oxygen on P10 and P13; (1 × 6 h) a single 6-h anesthetic exposure on P7, followed by control exposure to 30% oxygen on P10 and P13; (3 × 2 h) repeated 2-h sevoflurane exposures on P7, P10, and P13 for a cumulative 6 h of GA; or a control exposure to 30% oxygen on P7, P10, and P13. At P91, rats underwent behavioral testing using a spatial memory task, followed by euthanasia on P105-112. Behavioral results are not shown, as testing was interrupted by an unforeseen circumstance and as a result the rats displayed atypical learning curves (an increase in errors associated with the interruption in testing). Given this disrupted testing, we cannot draw any conclusions from the behavioral data.

Anesthesia

Sevoflurane was administered in a monitored anesthesia chamber *via* a calibrated flowmeter and agent-specific vaporizer. Chamber concentrations of carbon dioxide, oxygen, and sevoflurane were monitored throughout (VitalStore, Vetronic Services Ltd., Abbotskerswell, United Kingdom). Anesthesia was induced with 6% sevoflurane in 30% oxygen until loss of the righting reflex and response to toe and tail pinch, at which point exposure time commenced. Anesthesia was maintained by 2.5% sevoflurane in 30% oxygen. Rectal temperature was continuously monitored, and rats were kept normothermic at 36.5 ± 0.5°C throughout the experiment (PowerLab ADInstruments Ltd., Oxford, United Kingdom). Pulse oximetry was continuously monitored (VitalStore, Vetronic Services Ltd.), and oxygen saturation was maintained about 95% in all but two pups (see mortality data below). The respiratory rate and skin color were visually inspected every 20 min. Pups in the 6-h sevoflurane condition were removed from the chamber every 2 h and massaged to provide stimulation. Separate pups were used for transcardial arterial blood gas analysis (Radiometer ABL80, Cleveland, OH), which determined that all conditions produced mild hypercapnia. After anesthesia, the chamber was flushed with 30% oxygen until the pups recovered and displayed righting reflexes. The pups were then rubbed with bedding material from their home cage, and returned to their natural litter where they remained until weaning at P21. Mortality within group 1 × 6 h was 20% (2 of 10 pups). There was no mortality across the remaining experimental groups.

Tissue Processing and Perfusion

Twenty male rats (n = 5/condition) were euthanized at ~3.5 months of age (P105-112). Rats were deeply anesthetized with sodium pentobarbital 100 mg/kg and transcardially perfused with 1% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 1 min, followed by 4% paraformaldehyde in a 0.1 M PBS for 11 min. The brains were removed, postfixed at 4°C in 4% paraformaldehyde and 0.125% gluteraldehyde in PBS overnight, and coronally sectioned on a Vibratome (Leica VT1000S, Bannockburn, IL). Three blocks containing hippocampal CA1 were then manually selected from each animal and placed a 0.1 M PBS solution. The blocks then underwent cryo-protection in increasing concentrations of glycerol (from 10 to 30% in PBS), followed by a freeze-plunge.[14] In brief, blocks were rapidly immersed in liquid propane cooled to −190°C in a Universal Cryofixation System KF80 (Reichert-Jung, Vienna, Austria). The blocks were then immersed in 1.5% uranyl acetate and anhydrous methanol for 24 h at −90°C in an Automatic Freeze-Substitution System (Lecia, Vienna, Austria). The block temperature was then raised by 4°C/h to a temperature of −45°C at which point the blocks were washed with anhydrous methanol and infiltrated with Lowicryl resin (Electron Microscopy Sciences, Hatfield, PA). The resin was polymerized under ultraviolet light (360 nm) first for 48 h at −45°C, followed by 24 h at 0°C. The block faces were then trimmed to the stratum pyramidale and radiatum of CA1. Fifteen or more consecutive 90 nm ultrathin sections were cut with a diamond knife (Diatome, Bienne, Switzerland), and mounted on formvar/carbon-coated nickel slot grids (Electron Microscopy Sciences).

Morphologic Analyses

All imaging, synaptic, and mitochondrial analyses were performed by an experimenter blind to condition. Samples were

randomly recoded, and the code remained hidden until all morphologic analyses were complete. Serial section micrographs were taken with a Hitachi H-7000 transmission electron microscope (Hitachi High Technologies America, Inc., Pleasanton, CA) using a systematic-random approach. Sample size was guided by previous publications using the disector method for synaptic and mitochondrial analyses.[15–17] Five sets of 15 serial images were captured at ×17,000 using an AMT advantage CCD camera (Advanced Microscopy Techniques, Danvers, MA). To ensure the correct subregion was captured, all imaging was performed 95 to 105 μm from the pyramidal cell layer. Images were imported into Adobe Photoshop CS5 version 12.0.4 (Adobe Systems Inc., San Jose, CA).

Synaptic Analysis

Previous studies including rats from a similar study population have demonstrated that neonatal exposure to anesthesia induces marked hippocampal neurodegeneration and reductions in subicular synaptic volumetric density that persist into adulthood.[6,11,18] On this basis, synaptic density was selected as a measure of interest. Disector analysis was used to obtain a stereologically unbiased population of synapses for quantitative analysis. This methodology has been previously described.[15,16,19–21] In brief, all axospinous synapses were identified in the first two layers of each 15-section serial set. The postsynaptic density (PSD) was selected as the counting unit. PSD was defined by the presence of synaptic vesicles in the axon terminal and a distinct density in the postsynaptic dendritic spine.[16] Synapses were counted only if they were contained in the "reference" layer (section 1), but not in the corresponding "look-up" layer (section 2). This procedure was repeated for the middle two (sections 8 and 9) and last two (sections 14 and 15) images of each serial set. To increase sampling efficiency, the reference and look-up layers were reversed and the images were reanalyzed (fig. 1, A and B). The disector area was 49 μm^2 and the height of the disector was 180 nm. Axospinous synapse density was calculated as the total number of counted synapses from both images, divided by the total volume of the disector.

Postsynaptic Density Analysis

Postsynaptic density length was used as a surrogate measure for dendritic spine head size.[22] For each rat, 15 sets of three serial sections were analyzed, and all axospinous synapses in the second, middle section, were identified. Each synapse was followed through the three-image series, and the longest PSD was measured (fig. 1, C–E). For perforated synapses, the total length was reported as the sum of each PSD segment.[16] On average 200 PSDs were measured per rat.

Presynaptic Mitochondrial Analysis

For each 15-section series, the first five serial images were analyzed using the disector analysis described above. All presynaptic mitochondria were labeled and identified by the following criteria as: (1) electron dense and (2) enclosed within a bouton containing greater than 3 synaptic vesicles. Mitochondrial density (μm^3) was calculated as the total number of mitochondria present in the first reference layer and not the second look-up layer, divided by the total volume of the disector.

To control for a treatment effect on the raw number of boutons present, we analyzed the percentage of presynaptic terminals containing mitochondria. For each series, the eighth or middle section was used as a reference, and all boutons containing greater than 3 synaptic vesicles were identified and numbered. Each bouton was then followed throughout the series and the number of mitochondria recorded (fig. 2).[17] On average, 250 boutons were analyzed per rat. Boutons extending beyond 15 sections were included in the analyses so as not to preferentially exclude large boutons. The fraction of boutons containing mitochondria was calculated using Microsoft Excel (Microsoft Corporation, Redmond, WA).

Statistical Analysis

Linear fixed-effects analyses (*e.g.*, one-way ANOVA) cannot account for the dependence between multiple observations from the same animal. We therefore adopted a nested, multilevel approach.[23] Multilevel linear regression models were constructed to determine the main effect of treatment group on synaptic density, mitochondrial density, and the fraction of presynaptic terminals containing mitochondria. Random intercepts accounted for baseline variation and clustering between dams, animals, and series. To better evaluate for differences between experimental groups, *post hoc* pairwise comparisons with Bonferroni correction were performed. Additionally, the correlation between mean synaptic density and mean presynaptic mitochondrial density was evaluated using Pearson's correlation coefficient. To confirm that the observed relationship was not due to common group effects, data was centered on the grand mean and reanalyzed. Statistical significance of the above multilevel analyses and *post hoc* comparisons was defined as a P value of less than 0.05. Finally, differences in the cumulative distribution of PSD length were assessed in a pairwise manner *via* a two-sample Kolmogorov–Smirnov test. To reduce family-wise error, statistical significance was defined as a P value less than 0.0083 (or 0.05/6). All analyses were preformed using Stata version 11.2 (StataCorp, College Station, TX).

Results

Effects of Anesthesia on Synaptic Density

Neonatal exposure to anesthesia was associated with long-lasting reductions in mean CA1 synaptic density (fig. 3). Multilevel linear regression models controlling for baseline variation at the dam, animal, and series level determined that synaptic density differed based on the duration and number of sevoflurane exposures (table 1). As compared to control, a single 2-h exposure on P7 was not associated with alterations in synaptic density ($\beta = -0.078$, $P = 0.605$), while a single

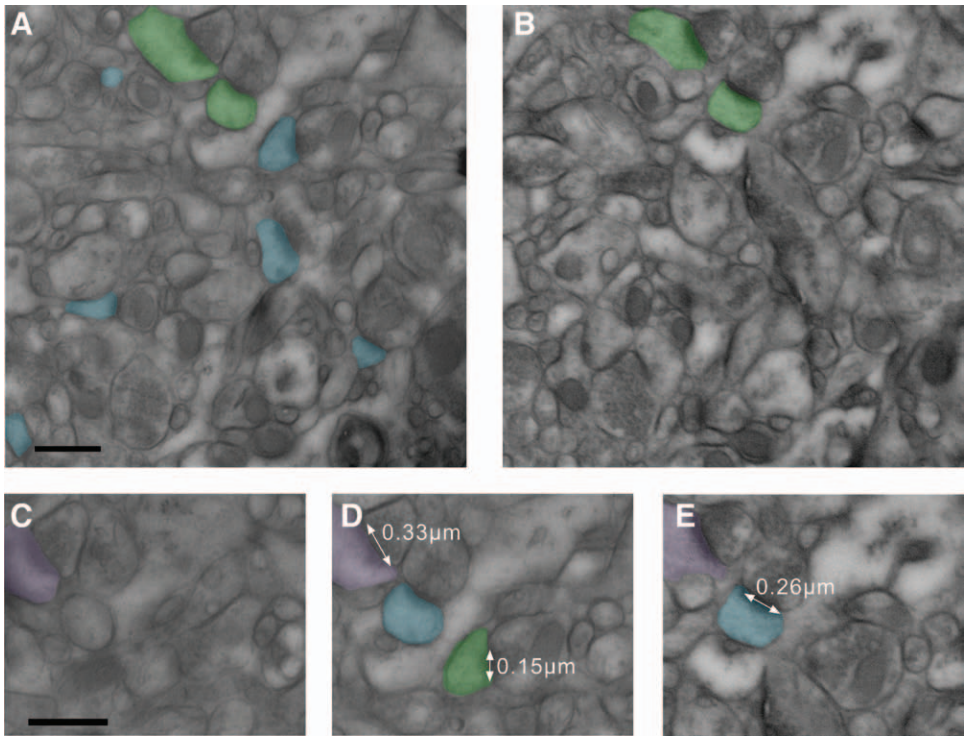

Fig. 1. (*A* and *B*) Electron micrographs demonstrating the disector method for synaptic density. *A* is considered the "reference" layer, *B* is considered the "look-up." Axospinous synapses present only in the reference layer, and not in the look-up are shaded in *blue*. Synapses present in both layers are shaded in *green*. *Scale bar*, 0.5 μm. (*C–E*) Electron micrographs demonstrating postsynaptic density (PSD) length analysis. All axospinous synapses in the middle layer (*D*) were identified. For each synapse, the longest PSD in any of the three serial sections was identified and measured. *Scale bar*, 0.5 μm.

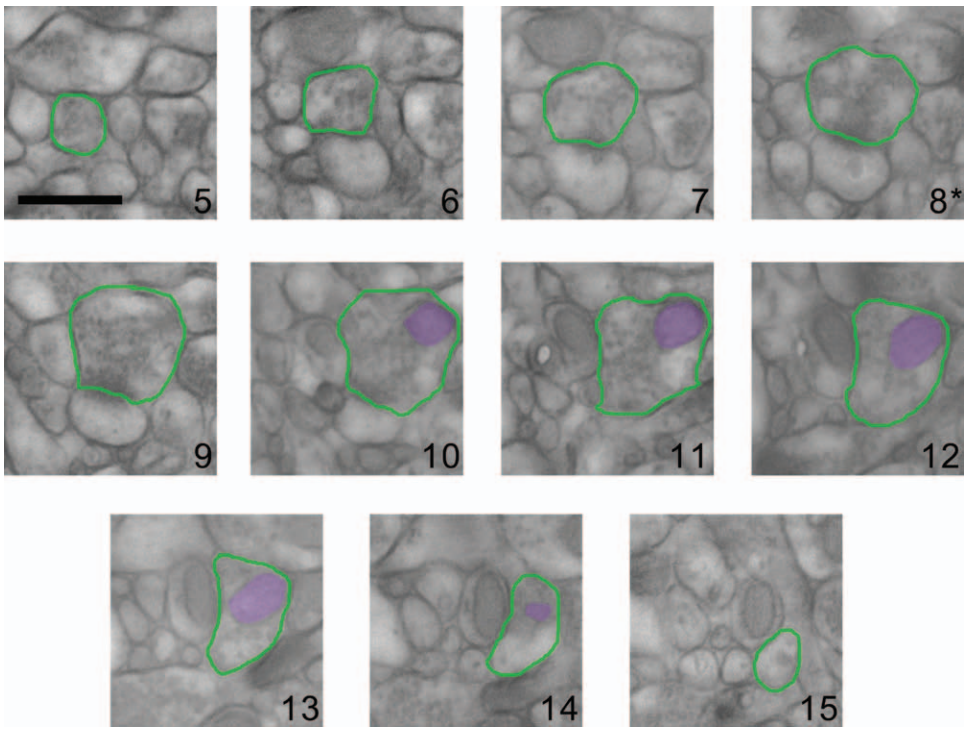

Fig. 2. Electron micrographs demonstrating the analysis of the fraction of boutons containing mitochondria. For each 15-layer series, the 8th or middle section (*) was used as the reference and all boutons identified. Each bouton (*green*) was then followed throughout the series, and the presence of mitochondria (*purple*) recorded. *Scale bar*, 500 μm.

6-h exposure yielded substantial synaptic losses (β = −0.336, P = 0.022). Furthermore, repeated 2-h exposures on P7, 10, and 13 were associated with a marked decrease in synaptic density (β = −0.681, P < 0.001).

In order to further evaluate for dose-related differences, *post hoc* pairwise tests using the Bonferroni correction were performed (table 2). As compared to a single 2-h exposure, both a single 6-h exposure on P7 and multiple 2-h exposures on P7, 10 and 13 resulted in significant decreases in mean synaptic density (P = 0.042 and P <0.001, respectively). Notably, multiple 2-h anesthetic exposures (of cumulative 6-h duration) resulted in greater synaptic loss as compared to a single 6-h exposure (P = 0.022).

Effects of Anesthesia on Presynaptic Density Length

Early exposure to sevoflurane was associated with a shift in the cumulative distribution of PSD length, a surrogate measure of dendritic spine size and morphology (fig. 4).[22] Pairwise two-sample Kolmogorov–Smirnov tests demonstrated that a single 2-h exposure resulted in smaller PSD lengths relative to a single 6-h exposure and multiple 2-h exposures (D = 0.086, P = 0.003 and D = 0.085, P = 0.006, respectively). However, no differences in PSD length were observed between a cumulative 6-h anesthetic exposure and control (multiple 2-h exposures D = −0.037, P = 0.615 and one 6-h exposure D = 0.053, P = 0.163, respectively, table 3). This suggests that while a single brief neonatal exposure induces a selective decrease in larger spines, repeated and prolonged exposures do not alter the distribution of spines.

Effects of Anesthesia on Presynaptic Mitochondrial Density

Disector analysis revealed a decrease in mean presynaptic mitochondrial density associated with longer cumulative durations of sevoflurane exposure (table 4). Relative to control, multilevel analysis revealed a slight, but nonsignificant decrease in mitochondrial density after a single 2-h exposure on P7 (β = −0.041, P = 0.864). Prolonged and repeated neonatal anesthetic exposure was associated with significant reductions in mean mitochondrial density. As compared to control, both a single 6-h exposure and multiple 2-h exposures were associated with a marked decrease in presynaptic mitochondrial density (β = −0.543, P = 0.023 and β = −0.748, P = 0.002, respectively). However, in contrast to the above synaptic data, repeated 2-h exposures did not result in a greater magnitude of mitochondrial loss relative to a single prolonged exposure (P = 0.156, table 2).

To determine if the observed reduction in mean presynaptic mitochondrial density was secondary to bouton loss, we calculated the fraction of boutons containing mitochondria. Multilevel linear regression confirmed that the anesthesia associated reductions in presynaptic mitochondrial density was independent of bouton-loss (table 4). As compared to control, a single 6-h and multiple 2-h exposures to sevoflurane resulted in a decreased fraction of boutons containing mitochondria (β = −0.107, P < 0.001 and β = −0.116, P = 0.001, respectively). *Post hoc* analysis did not reveal a significant difference between these two cumulative 6-h exposures (P = 0.770).

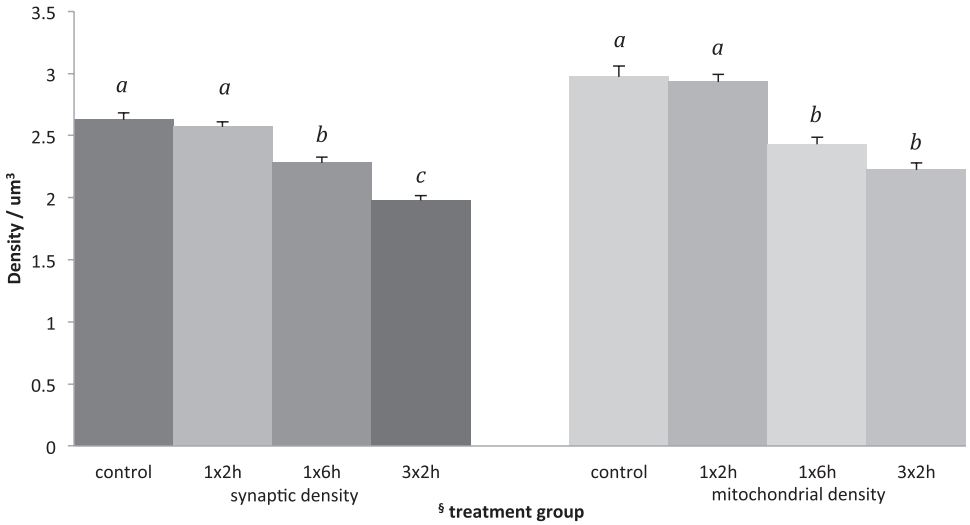

Fig. 3. Mean synaptic and mitochondrial density by treatment group: multiple and prolonged exposure to anesthesia was associated with greater reductions in both synaptic and mitochondrial density. Synaptic and mitochondrial density are expressed as mean ± SEM. §Treatment group (n = 5/group): 1×2 h = single 2-h exposure postnatal day 7 (P7); 1×6 h = single 6-h exposure (P7); 3×2 h = three 2-h exposures (P7, P10, and P13). *a*, *b*, *c* indicates groups with differing superscripts (*a*, *b*, or *c*) are significantly different from one another (statistical significance defined as P < 0.05). For example, for synaptic density: the control and 1×2-h groups do not differ from each other, but both differ from the 1×6-h and 3×2-h groups, which also differ from one another.

Table 1. Multilevel Linear Regression for Synaptic Density

Variable	Coefficient	95% CI	*P* Value*
Treatment group†			
1×2 h	−0.078	−0.371 to 0.216	0.605
1×6 h	−0.336	−0.623 to −0.049	0.022
3×2 h	−0.681	−0.972 to −0.390	<0.001
Control	2.630	2.415 to 2.845	—

Level 1 (dam) n = 6; level 2 (animal) n = 20; and level 3 (series) n = 100.

*Statistical significance as defined by $P < 0.05$. †Treatment group: 1×2 h = single 2-h exposure postnatal day 7 (P7); 1×6 h = single 6-h exposure (P7); and 3×2 h = three 2-h exposures (P7, 10, and 13).

Correlation between Synaptic Loss and Mitochondrial Density

Analysis with Pearson's correlation coefficient demonstrated a statistically significant relationship between the mean synaptic density and the mean presynaptic mitochondrial density ($r = 0.750$, $P < 0.001$; fig. 5). To control for common group effects, the correlation between synaptic and mitochondrial density was reevaluated using mean-centered data from each group. Results remained significant ($r = 0.500$, $P = 0.025$), and suggest a possible relationship between the alterations in mitochondrial localization and observed synaptic losses.

Overall, these findings indicate that relative to a single prolonged exposure, repeated neonatal exposure to anesthesia is highly neurotoxic, and results in greater ultrastructural damage. Synapses appear to be particularly vulnerable, and brief but repeated exposure results in marked and long-lasting synaptic loss. In contrast, mitochondrial toxicity is best predicted by the cumulative duration of exposure, not the total number of exposures.

Discussion

In this study, we demonstrate that neonatal sevoflurane exposure produces long-lasting and dose-related ultrastructural damage. This damage is characterized by synaptic loss, decreased presynaptic mitochondrial localization, and a shift in the distribution of PSD length. Of note, the magnitude of synaptic loss induced by three 2-h exposures was greater than that of a single 6-h exposure of equivalent total length. Conversely, animals exposed to a cumulative 6 h of anesthesia had marked alterations in mitochondrial localization that were independent of the total number of exposures.

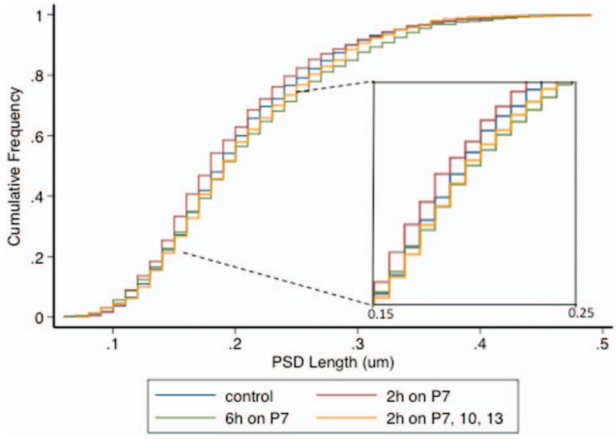

Fig. 4. Cumulative frequency distribution: measurements revealed a dose-related shift in postsynaptic density (PSD) length. A single 2-h exposure on postal day 7 (P7) was associated with a decrease in larger head-bearing spines. The detail is magnified and shown in the *inset*.

These findings provide a potential histological correlate for human population-based studies demonstrating that children with repeated exposures to anesthesia are at increased risk of attention-deficit/hyperactivity disorder and cognitive impairment.[1,3–5] One study by Flick *et al.*[3] demonstrated a 2.12-fold increased risk of learning disability associated with multiple, but not single, early exposures. The risk of learning disability has also been shown to increase with longer cumulative durations of anesthetic exposures.[1] Nonetheless, these retrospective studies are limited in their ability to control for surgical stress, perioperative inflammation, hypoxia, and comorbidity. The present study therefore provides an important and experimentally controlled model to further investigate these observations.

This is the first study of which we are aware to characterize the long-term ultrastructural effects associated with multiple early anesthetic exposures. We previously demonstrated that relative to a single exposure, adult rats with multiple early isoflurane exposures had greater impairment on a spatial working memory task.[13] Our work builds on prior literature showing that even a single early exposure to *N*-methyl-D-aspartate antagonists or gamma aminobutyric acid mimetics is immediately neurotoxic and triggers widespread apoptotic neurodegeneration.[24,25] Of note, a 6-h neonatal exposure to GA has been shown to induce a 21-fold increase in

Table 2. *Post Hoc* Pairwise Comparisons with Bonferroni Correction

		Synaptic Density		Mitochondrial Density		Fraction of Boutons with Mitochondria	
Treatment Groups*		χ^2	*P* Value†	χ^2	*P* Value†	χ^2	*P* Value†
1×2 h *vs.*	1×6 h	3.03	0.042	4.41	0.036	11.50	<0.001
	2×3 h	16.98	<0.001	8.76	0.003	13.56	<0.001
1×6 h *vs.*	2×3 h	5.31	0.022	0.74	0.156	0.09	0.770

*Treatment groups: 1×2 h = single 2-h exposure postnatal day 7 (P7); 1×6 h = single 6-h exposure (P7); and 3×2 h = three 2-h exposures (P7, 10, and 13). †Statistical significance as defined by $P < 0.05$.

Table 3. Kolmogorov–Smirnov Tests

Treatment Groups*		*D*	*P* Value†
Control *vs.*	1×2 h	0.062	0.056
	1×6 h	0.053	0.163
	3×2 h	0.037	0.615
1×2 h *vs.*	1×6 h	0.086	0.003†
	3×2 h	0.085	0.006†
1×6 h *vs.*	3×2 h	0.036	0.694

*Treatment groups: 1×2 h = single 2-h exposure postnatal day 7 (P7); 1×6 h = single 6-h exposure (P7); and 3×2 h = three 2-h exposures (P7, 10, and 13). †Statistical significance as defined by $P < 0.0083$.

hippocampal CA1 neuronal degeneration.[6] Lunardi *et al.* subsequently demonstrated that rats with a single 6-h exposure to a cocktail of midazolam, nitrous oxide, and isoflurane on P7, had long-lasting reductions in synaptic density and the number of multiple synaptic boutons. Here we further establish that relative to a single exposure, brief but repeated exposures are associated with even greater long-term synaptic losses.

Dendritic spine morphology is thought to be closely related to synaptic function.[26] PSD length and spine head volume are directly correlated with synaptic strength, the number of receptors, and the availability of presynaptic docked vesicles.[16,22,27,28] Briner *et al.*[9] previously demonstrated that rats exposed to propofol on P10 had lower numbers of mature prefrontal cortex spines with a head diameter between 0.3 and 0.4 μm. Similarly, we found that a single 2-h exposure to sevoflurane on P7 is associated with a selective decrease in larger head-bearing spines. However, longer 6-h exposures did not shift the cumulative distribution of spine types. In the setting of a decreased spine density, this suggests that brief neonatal anesthetic-exposures decrease the stability of large spines, while prolonged exposure causes a more uniform pattern of neurodegeneration, and is unrelated to spine-type or maturity. This is of note, as it demonstrates that even short exposures to GA may induce long-term alterations in neuronal circuitry.

Impaired mitochondrial function and morphogenesis has been suggested as a mechanism for anesthetic neurotoxicity and long-term cognitive impairment. Mitochondria are dynamic organelles, critical to synaptogenesis, plasticity,

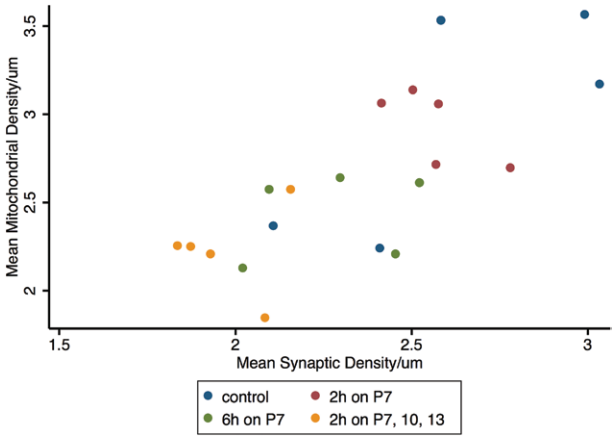

Fig. 5. Scatter plot of mean presynaptic mitochondrial density by mean synaptic density.

calcium buffering, and vesicle recruitment.[29–31] Neonatal exposure to general anesthetics has been shown to alter mitochondrial fission/fusion and activate the intrinsic, mitochondrial-dependent, apoptotic cascade.[12,32,33] A recent study by Sanchez *et al.*[10] showed persistent alterations in mitochondrial morphology, function, and presynaptic localization after a single 6-h neonatal exposure to anesthesia. Our results similarly demonstrate long-lasting reductions in presynaptic mitochondrial density associated with longer cumulative exposures to sevoflurane. In the context of our synaptic data, this suggests a "threshold effect" for GA-induced neurotoxicity. It is possible that early alterations in mitochondrial trafficking or fission sensitize surviving synapses to subsequent exposures, and may in part explain the clinical effects observed with multiple exposures.

The neurotoxicity associated with general anesthesia is age dependent, and peak toxicity occurs during periods of rapid neurodevelopment and synaptogenesis.[25,34] In humans, this period extends from the third trimester to several years postnatally, and up until P14 in rats. In the current study, rat pups were exposed to GA between P7 and P13, a timeframe thought to approximate the brain growth spurt occurring at birth in humans.[35] More conservative neuroinformatics models estimate that P7-10 is the neurodevelopmental equivalent of premature human infants born in

Table 4. Multilevel Linear Regression for Presynaptic Mitochondrial Density and the Fraction of Boutons Containing Mitochondria

	Mitochondrial Density			Boutons with Mitochondria		
Variable	Coefficient	95% CI	*P* Value*	Coefficient	95% CI	*P* Value*
Treatment group†						
1×2 h	−0.041	−0.509 to 0.428	0.864	0.000	−0.062 to 0.062	0.999
1×6 h	−0.543	−1.011 to −0.074	0.023	−0.107	−0.168 to −0.045	0.001
3×2 h	−0.748	−1.217 to −0.280	0.002	−0.116	−0.178 to −0.054	<0.001
Control	2.972	2.641 to 3.303	<0.001	0.595	0.551 to 0.638	<0.001

Level 1 (dam) n = 6; level 2 (animal) n = 20; and level 3 (series) n = 100.

*Statistical significance as defined by $P < 0.05$. †Treatment group: 1×2 h = single 2-h exposure postnatal day 7 (P7); 1×6 h = single 6-h exposure (P7); and 3×2 h = three 2-h exposures (P7, 10, and 13).

the late-third trimester.[36–38] Nevertheless, these infants are at high risk of morbidity and mortality, and often require early and repeated exposure to anesthesia. Clarifying the neurohistologic impact of multiple anesthetic exposures is therefore essential for clinical decision-making and the development of pharmacological intervention.

Our research has several limitations. First, the number of rats included in each experimental group was low (n = 5/group). Although such a sample size is comparatively large for stereologic electron microscopy studies, we may still be underpowered to detect more subtle differences or shifts in PSD length.[15–17] Second, we cannot address whether there are any sex-related differences, as all of the animals were male. This is significant, as prior studies have reported that adult females with single postnatal anesthetic-exposures have greater deficits in spatial working memory, while a more recent study demonstrated long-term cognitive dysfunction in male, but not female rats.[13,39,40] Finally, our study employs a nonsurgical experimental model. Noxious stimulation itself causes marked neurodegeneration, and prior research suggests that sedation with ketamine may actually protect neonates from pain-related neurotoxicity.[41,42] It will be important in future work to utilize models that incorporate a surgical stimulus and examine effects in both males and females.

In conclusion, repeated neonatal exposure to GA is associated with greater long-term reductions in both synaptic density and presynaptic mitochondrial localization. Furthermore, a single 2-h anesthetic exposure shifts the distribution of PSD length, and alters dendritic spine morphology. This suggests a "threshold effect" for GA-induced neurotoxicity, such that even though brief exposures induce long-term alterations in neuronal circuitry, longer exposures affect mitochondrial localization and sensitize surviving synapses to subsequent loss.

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Competing Interests

The authors declare no competing interests.

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