Noninvasive Tracking of Anesthesia Neurotoxicity in the Developing Rodent Brain

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ABSTRACT

Background: Potential deleterious effect of multiple anesthesia exposures on the developing brain remains a clinical concern. We hypothesized that multiple neonatal anesthesia exposures are more detrimental to brain maturation than an equivalent single exposure, with more pronounced long-term behavioral consequences. We designed a translational approach using proton magnetic resonance spectroscopy in rodents, noninvasively tracking the neuronal marker N-acetyl-aspartate, in addition to tracking behavioral outcomes.

Methods: Trajectories of N-acetyl-aspartate in anesthesia naïve rats (n = 62, postnatal day 5 to 35) were determined using proton magnetic resonance spectroscopy, creating an "N-acetyl-aspartate growth chart." This chart was used to compare the effects of a single 6-h sevoflurane exposure (postnatal day 7) to three 2-h exposures (postnatal days 5, 7, 10). Long-term effects on behavior were separately examined utilizing novel object recognition, open field testing, and Barnes maze tasks.

Results: Utilizing the N-acetyl-aspartate growth chart, deviations from the normal trajectory were documented in both single and multiple exposure groups, with z-scores (mean ± SD) of –0.80 ± 0.58 (P = 0.003) and –1.87 ± 0.58 (P = 0.002), respectively. Behavioral testing revealed that, in comparison with unexposed and single-exposed, multiple-exposed animals spent the least time with the novel object in novel object recognition ($F_{(2,44)}$ = 4.65, P = 0.015), traveled the least distance in open field testing ($F_{(2,57)}$ = 4.44, P = 0.016), but exhibited no learning deficits in the Barnes maze.

Conclusions: Our data demonstrate the feasibility of using the biomarker N-acetyl-aspartate, measured noninvasively using proton magnetic resonance spectroscopy, for longitudinally monitoring anesthesia-induced neurotoxicity. These results also indicate that the neonatal rodent brain is more vulnerable to multiple anesthesia exposures than to a single exposure of the same cumulative duration. **(Anesthesiology 2018; 129:118-30)**

CLINICAL concern and debate remain, regarding the potential harmful effects of anesthesia in the developing brain, thought to be especially vulnerable because of its inherent enhanced neuroplasticity.[1–3] Several studies suggest children having two or more anesthetic exposures are at increased risk of learning disabilities.[4,5] Paralleling this, in animal models, neonatal rats show more severe neuronal damage after multiple exposures than after a single exposure.[6,7] However, preclinical studies have not investigated long-term neuronal sequela, or potential for recovery, as experimental methods for documenting tissue damage are terminal procedures.

To validate these reports clinically, a robust and specific biomarker is required, one which can track potential harmful effects to the human brain noninvasively. Currently, brain development in children is evaluated clinically through secondary observational assessments, such as motor, language, and developmental milestones. These serve as indicators of normal or substandard brain development, allowing the potential to address developmental abnormalities.[8,9] Though clinically useful, these milestones rely on subjective and poorly quantifiable criteria. An unbiased, quantitative approach, using a surrogate biomarker, would allow for an objective evaluation.

What We Already Know about This Topic

- A noninvasive biomarker of the impact of anesthetics on the neonatal brain is currently unavailable.
- N-acetyl-aspartate is an amino acid that is abundantly present in neurons and is a biomarker of brain development. Whether the trajectory of N-acetyl-aspartate levels, as measured by proton magnetic resonance spectroscopy, can be used as a marker of anesthetic neurotoxicity in the developing brain is not known.

What This Article Tells Us That Is New

- With either a single or multiple exposure to sevoflurane, a reduction in N-acetyl-aspartate was observed; this reduction led to a deviation in the normal trajectory in the developing brain. Effects of multiple anesthesia exposures with sevoflurane were more pronounced than a single exposure on both N-acetyl-aspartate trajectory and animal behavior.
- The results suggest that N-acetyl-aspartate may serve as a biomarker of the impact of anesthetic agents on the developing brain; however, further validation of its utility is necessary.

Proton magnetic resonance spectroscopy enables longitudinal measures in the same subject over time, such as during brain development[10–12] or disease progression.[13–15]

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For instance, N-acetyl-aspartate, measured noninvasively in the live brain, is an excellent biomarker of brain development,[16,17] and changes in parallel with decreases in grey matter volume and increases in white matter volume.[18,19] We previously demonstrated that N-acetyl-aspartate trajectories can track rodent brain maturation, and documented aberrant short-term N-acetyl-aspartate changes from postnatal days 8 and 9 after a single prolonged anesthesia exposure on postnatal day 7.[20] Here, we hypothesize that long-term trajectories of N-acetyl-aspartate (postnatal days 5 to 35) will serve as a robust biomarker of normal rodent brain development, and thereby, function as reference, allowing for documenting deviations from normal growth. Specifically, we propose that N-acetyl-aspartate brain growth profiles could be used similarly to clinical growth charts that are applied to track height and weight percentiles in infants and children.[21–23] Growth charts, constructed by measuring normal infants and children at frequent intervals, use derived trajectories as references for tracking normal and substandard growth in the background of continual increases in these measured parameters.

We, therefore, applied proton magnetic resonance spectroscopy to measure the concentration of N-acetyl-aspartate over time, tracking normal and abnormal brain development in rodents, with the ultimate goal of defining a noninvasive, unbiased, and objective criterion to identify long-term sequela of neonatal anesthesia exposure(s). We aimed to first evaluate N-acetyl-aspartate as a surrogate biomarker of rat brain development; second, to create an N-acetyl-aspartate growth chart characterizing normal brain development; and finally, to use the growth chart to track brain maturation in rat pups after single or multiple exposures to sevoflurane anesthesia. In addition, we documented behavioral outcomes in parallel series of experiments with rats exposed to the same anesthesia regimens. Our results show that, though the cumulative duration of the multiple exposures was equal to that of the single exposure, rat pups with multiple exposures have more severe deviations from normal N-acetyl-aspartate growth trajectories and behavior parameters.

Materials and Methods

All animal procedures were approved by the local institutional animal care and use committees at Stony Brook University (Stony Brook, New York) and Brookhaven National Laboratory (Upton, New York). Lactating Sprague-Dawley dames with male pups were ordered from Taconic Biosciences (USA); only male neonatal rats were used for testing purposes to increase the effect size, as oxytocin and estradiol have been demonstrated to decrease vulnerability of the developing brain to the effects of anesthesia.[24–27]

Experimental Groups for Proton Magnetic Resonance Spectroscopy

N-acetyl-aspartate Growth Chart Group. The first group (table 1) comprised 62 anesthesia naïve animals ranging in age from postnatal day 5 to 35. The purpose of this group was to create the rat brain N-acetyl-aspartate growth chart specific to the thalamus. For this purpose, we acquired spectra using proton magnetic resonance spectroscopy in each rat pup and plotted N-acetyl-aspartate as a function of age. To acquire the spectra, animals were anesthetized only during the one short scan they each had, as described in "Anesthesia Exposure(s)." All animals were euthanized after the scan. Using a sigmoidal regression model, the link between N-acetyl-aspartate in the thalamus and animal age was quantified based on the proton magnetic resonance spectroscopy data obtained, thereby deriving the growth trajectory.

Anesthesia Exposure Groups. The "single *versus* multiple anesthesia" exposure proton magnetic resonance spectroscopy study was designed according to table 1, where animals were randomly assigned to the groups. Specifically, we implemented a single long anesthesia exposure (group 2) and a multiple short anesthesia exposure group (group 3). For group 2, a single anesthesia exposure was performed on postnatal day 7 for 6 h, while group 3 rat pups had three different anesthesia exposures on postnatal days 5, 7, and 10, each lasting 2 h, for a total of 6 h of exposure. In both groups, proton magnetic resonance spectroscopy spectra were acquired from

Table 1. Experimental Groups

Group	Experimental Objective	Age(s) at Exposure	Duration of Each Exposure	Age at 1HMRS Acquisition	Age at Behavioral Testing
1 (n = 62)	Create NAA growth chart	n/a	Only during 1HMRS acquisition	PND 5–35	n/a
2 (n = 9)	Single, long exposure + 1HMRS	PND 7	6 h	1, 2, 3, and 4 weeks	n/a
3 (n = 5)	Multiple, short exposures + 1HMRS	PND 5, 7, 10	2 h	1, 2, 3, and 4 weeks	n/a
4 (n = 20)	Control group (unexposed) + behavior	n/a	0 h	n/a	4–6 weeks
5 (n = 20)	Single, long exposure + behavior	PND 7	6 h	n/a	4–6 weeks
6 (n = 20)	Multiple, short exposures + behavior	PND 5, 7, 10	2 h	n/a	4–6 weeks

1HMRS = proton magnetic resonance spectroscopy; NAA = N-acetyl-aspartate; PND = postnatal day.

the thalamus during the anesthesia exposure on postnatal day 7, and then again at 2, 3, and 4 weeks of age. The thalamus was chosen based on several considerations. First, the thalamus is reported to be one of the brain regions showing the highest level of anesthesia-induced neurotoxicity.[2] Second, the thalamus is a large and centrally positioned brain structure, which is advantageous from the point of view of proton magnetic resonance spectroscopy, as it is farthest away from the skull and overlying subcutaneous fat and skin, which can create artifacts during measurements. Finally, anatomically, the thalamus allows for capture of both hemispheres simultaneously, doubling the voxel size, therefore increasing the single-to-noise ratio for the data acquired. Figure 1 presents the experimental design for these two groups.

Experimental Groups for Behavior

The experimental groups undergoing behavioral testing after anesthesia exposures were also randomly assigned, as outlined in table 1. In the behavioral groups, the rat pups in the single long exposure group had 6 h of sevoflurane anesthesia on postnatal day 7 (group 5) and, those in the multiple short exposure group (group 6) had three anesthesia exposures on postnatal days 5, 7, and 10, each lasting 2 h, for a total of 6 h of exposure time, while the control group (group 4) did not have any exposures to anesthesia. Though no *a priori* statistical power calculation was conducted for this study, based on our previous experience with the behavioral assays used, we designed the experiment with 20 animals/group to achieve reasonable statistical power. Animals were later tested for behavior between 4 to 6 weeks of age, by an experimenter blinded to the animal groups (table 1). The order of behavioral testing was randomized, so that half of the animals in each group had Barnes maze testing before open field and novel object testing, while the other half had open field and novel object testing before Barnes maze testing.

Anesthesia Exposure(s)

During all anesthesia exposures, the animals were first induced with 5% sevoflurane in 100% oxygen using an induction chamber. Once the righting reflex was suppressed, the animals were moved to a heating pad with anesthesia maintained at 2.2% sevoflurane delivered in a 1:1 mixture of air and oxygen, providing a fraction of inspired oxygen level at 60%. Throughout the exposure, animals were monitored

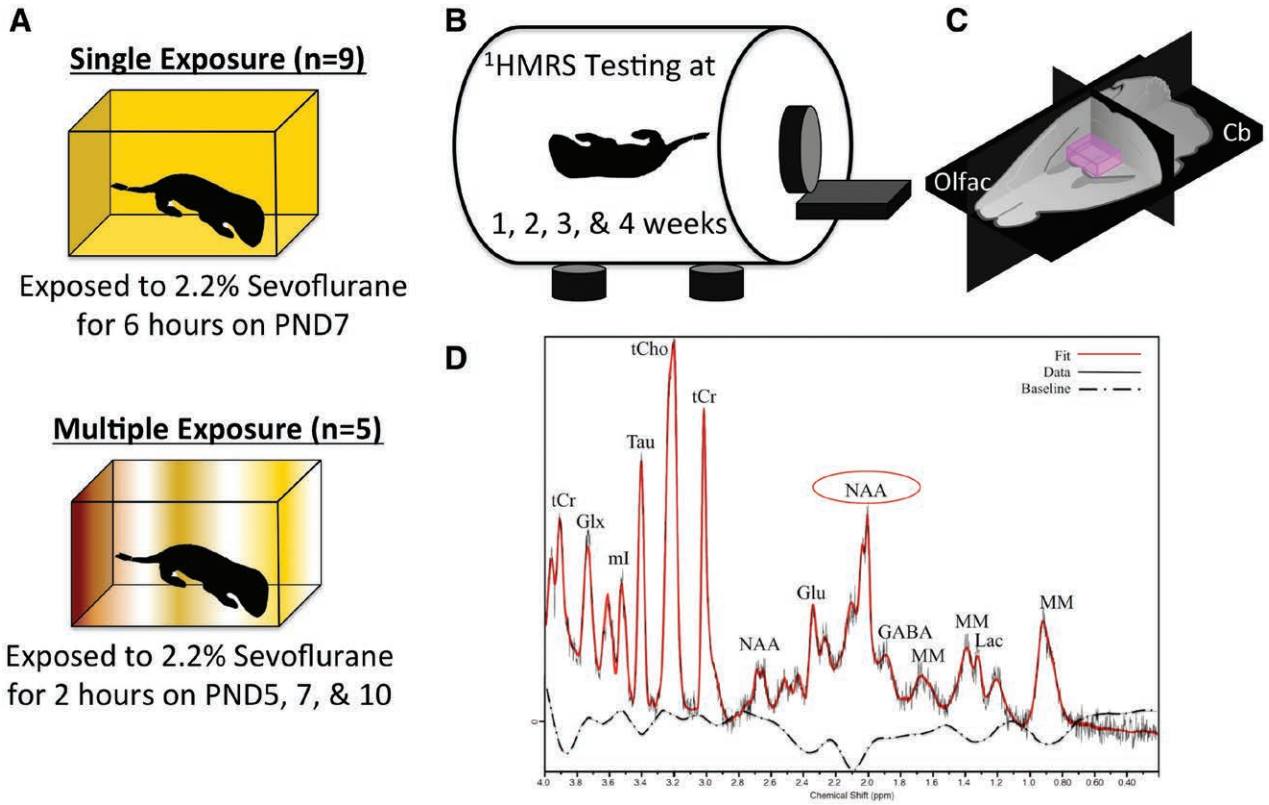

Fig. 1. Experimental design for the exposure groups of the proton magnetic resonance spectroscopy (1HMRS) portion of testing. (*A*) Animals were divided into two anesthesia exposure groups, single exposure and multiple exposure, with the total duration of anesthesia being 6 h for both. (*B*) Each animal underwent repeated testing using 1HMRS at 1, 2, 3, and 4 weeks of age. (*C*) The thalamus was identified using three orthogonal planes, and a voxel was placed over the thalamus of both hemispheres. (*D*) Spectrum were then acquired and processed to provide the concentration of NAA in the live animal brain. Cb = cerebellum; GABA = gamma amino butyric acid; Glu = glutamate; Glx = glutamate and glutamine; Lac = lactate; mI = myoinositol; MM = macromolecules; NAA = N-acetyl-aspartate; Olfac = olfactory bulb; PND = postnatal day; Tau = taurine; tCho = total choline; tCr = total creatinine.

for heart rate, respiratory rate, oxygen saturation, and body temperature, which was maintained at 37° ± 1°C. Minor adjustments in sevoflurane and oxygen concentrations were made to maintain the following physiologic parameters: (1) respiratory rate 60 to 70 breaths/min; (2) heart rate between 300 to 400 beats/min; and (3) oxygen saturation greater than 90%. Upon completion of anesthesia exposure, the rats emerged from anesthesia and recovered before being returned to the lactating dame. An animal was deemed "recovered" once it regained the righting reflex.

Proton Magnetic Resonance Spectroscopy and Anesthesia

Animals were anesthetized with sevoflurane for all of the proton magnetic resonance spectroscopy acquisitions. Proton magnetic resonance spectroscopy spectra were acquired using a small animal 9.4 Tesla magnetic resonance imaging instrument interfaced to a Bruker Advance console, and controlled by Paravision 5.0 software (Bruker Biospin Corp, USA), as previously described.[20] Sevoflurane was used for anesthesia during scanning, with optical magnetic resonance imaging compatible monitors (SA Instruments, Inc., USA) for heart rate, respiratory rate, and oxygen saturation. Body temperature was controlled using a computer-assisted heating system (SA Instruments, Inc.) to maintain normal temperature. An 11.2-cm radio frequency volume coil was used for signal transmission, and a custom built 3-cm surface radio frequency coil was received for signal reception. Animals were imaged in the supine position, with the thalamic area of the brain positioned in the center of the magnet. The centering of the thalamic region was confirmed by anatomical images acquired in three orthogonal planes (coronal, axial, and sagittal) using a rapid acquisition with relax enhancement sequence with the following parameters: repetition time = 2500 ms; echo time = 40 ms; number of averages = 2; rapid acquisition with relaxation enhancement factor = 8; number of slices = 25; in plane resolution = 0.117 mm/pixel; slice thickness = 0.9 mm; and slice gap = 0.1 mm. A voxel size of 3 × 2 × 3 mm was placed over the thalamic region covering both hemispheres. MAPSHIM (software package for Paravision) was used to apply both first- and second-order shims to minimize B_0 inhomogeneity in the voxel. Proton magnetic resonance spectroscopy was acquired using a point-resolved spectroscopy sequence (repetition time = 4,000 ms; echo time = 12 ms; number of averages = 1,024; spectral width = 8,012 Hz; number of acquired complex points = 4,096; and scan time ≈ 69 min). For each scan, the free induction decay signal was recorded separately, and custom software written in MATLAB (The Math Works, Inc., USA) was used to correct frequency and phase changes during the scan. A water unsuppressed scan was acquired using the same magnetic resonance parameters used for the water suppressed scan as an internal reference standard to quantify the metabolite concentrations. This process was repeated for each proton magnetic resonance spectroscopy acquisition.

Spectral Processing

LCModel software (Stephen Provencher Inc., Canada) was used for spectral processing and analysis as previously described.[20] Briefly, the spectra were fitted using a set of 18 simulated metabolites to best fit spectra with overlapping peaks between metabolites. Brain water concentration was adjusted for age given the higher water concentration in the brains of neonatal animals.[10–12] Using the signal-to-noise ratio and the full width half maximum generated by LCModel analysis, we excluded spectra of poor quality, specifically excluding spectra with an signal-to-noise ratio of 8 or less, and a full width half maximum of more than 0.040 ppm. Because we concentrated solely on N-acetyl-aspartate, the Cramér–Rao lower bounds identifying the accuracy of the fit were less than 10% for all our data. Based on the aforementioned mentioned LCModel criteria, one animal from each of the two exposure groups (groups 2 and 3) were excluded from the analysis. Table 1 presents the final number of animals used after all exclusions.

Behavior Testing

Three main behavioral assays were used for the study: the novel object recognition task, the Barnes maze, and the open field test. The total number of animals for behavioral testing was 60, with 20 animals in each group (table 1). While both the novel object recognition and the Barnes maze assays are affected by damage to temporal lobe structures, especially hippocampal and parahippocampal structures, the novel object recognition requires episodic memory, since the animal must remember two objects found in a particular temporal and spatial context; whereas, the Barnes maze is an aversively motivated task that requires the animal to use primarily extramaze cues to locate the position of a fixed escape box.

For the novel object recognition test, a custom built 1.2 m^2 wooden open field setup with 50-cm high walls was used. Two objects were placed at specific locations in the apparatus. The animals were then placed in the apparatus for two 10-min sessions on the first day of testing. On the subsequent day, one of the objects was replaced with a novel object and the animals were again placed in the apparatus and allowed to explore both objects for 5 min. The time spent exploring each object was quantified, with the primary parameter being the time spent with the novel object as a fraction of time spent with both objects.

For the open field test, the same 1.2 m^2 field as described for novel object recognition was used. Animals were placed in the apparatus for 10 min and their movements were recorded. Data was collected and analyzed using ANY-maze software (Stoelting Co., USA) to quantify proximity to the wall, average distance traveled by the animal, and the number of lines crossed.

The Barnes maze (San Diego Instruments, Inc., USA) consisted of a circular surface, 120 cm in diameter, with 20 holes that were each 10 cm in diameter, equally spaced

along the circumference. A hidden escape box was placed under one of the holes, with visual cues placed around the table for visual-spatial recognition. A camera was placed above the maze, connected to a computer with ANY-maze software for analyzing animal movements and behavior. Animals were given two training sessions each day for four consecutive days of training on the maze, allowing them to learn the location of the escape box, using visual-spatial cues. If they were unable to find the escape box after 150 s, they were guided there. Once they reached the escape box, they were left there for about 30 s. After each individual animal trial, 10% bleach was used to clean the surface of the maze and the escape box. On the fifth day, probe trials were performed, where the escape box was removed, and time spent in the target quadrant of the maze was the primary outcome measure for this test, reflecting memory of escape box location.

Additionally, home cage testing was performed on five animals in each of the animal behavior groups, to identify possible gross motor deficits. These animals were tested for general motor activity over the course of 22 h, divided into 30-min bins, with times correlating with light and dark phases. Total light phase activity was noted as the total number of beam breaks during the light phase, and total dark phase activity was noted as the number of beam breaks during the dark phase.

Statistical Analysis

In order to develop the N-acetyl-aspartate growth curve, animals in group 1 were analyzed. Sigmoidal regression was used to quantify the association between N-acetyl-aspartate in the thalamus and the age of the rats.

To compare the N-acetyl-aspartate in the *single-exposed* and *multiple-exposed* groups at each of the first 4 weeks of life, a z-score was estimated for each animal at each testing time point using the following formula:

$$\text{z-score} = \frac{NAA\,value - predicted\,value\,of\,NAA\,at\,age\,K}{Standard\,error\,of\,predicted\,of\,NAA\,at\,age\,K}$$

A two-sided *t* test was used to investigate at each week if the average z-score of the single-exposed group and the multiple-exposed group were significantly different from zero, respectively. A two-sided *t* test for independent groups was also used to investigate at each week, if there was difference in average z-scores between two groups. We control for family-wise error rate *via* Bonferroni correction and consider a *P*-value less than 0.05/4 = 0.0125 as statistically significant. This analysis was conducted using R version 3.0.0.

For statistical analysis of behavioral tests, ANY-maze software was used. The three groups were compared using ANOVA testing for each of the outcome measures tested. This was followed with a *post hoc* test using the Fisher least significant difference test to determine which groups were different.

Results

All rats demonstrated similar weight gain patterns, with no differences found between the multiple anesthesia groups, single long exposure, and control groups.

Proton Magnetic Resonance Spectroscopy

N-acetyl-aspartate in Anesthesia Naïve Animals (Group 1). The relationship between N-acetyl-aspartate and animal age was determined by applying a sigmoidal regression model to the proton magnetic resonance spectroscopy data obtained using group 1 animals (table 1). The sigmoidal model describes the trajectory of N-acetyl-aspartate in the developing rat brain, using three parameters. The first parameter, *a*, was used to approximate the maximal amount of N-acetyl-aspartate in the rat brain. The second parameter, x_0, was used to estimate the age that reaches half of the maximum. The third parameter, *b*, represents growth rate. The model was fitted using function *nls* under R version 3.0.0. The model derived is described as follows:

$$\text{N-acetyl-aspartate} = \frac{6.82}{1 + e^{-\frac{Age - 10.75}{4.67}}}$$

The estimated regression coefficient (± standard error of the estimate) for *a*, *b*, and x_0, were 6.82 ± 0.19, 4.67 ± 0.49, and 10.75 ± 0.44, respectively. The R^2 of the model was 0.91, signifying that 91% variation of the mean N-acetyl-aspartate can be explained by the model. Figure 2A shows the individual data, the fitted value, and the 95% CI of the sigmoidal regression model. The fitted value of this model was then further qualified by the 50, 75, 90 and 95% prediction intervals (fig. 2B). These prediction intervals correlate to z-scores of ±0.5, ±1, ±1.5, and ±2, respectively. The model shows a continuous increase in N-acetyl-aspartate on a day-to-day basis during postnatal days 5 to 35. Note that the rate of increase is faster during the first 20 days of life and slower between 20 and 35 days.

N-acetyl-aspartate in Anesthesia-exposed Animals (Groups 2 and 3). Based on the N-acetyl-aspartate growth curve derived from the anesthesia naïve rat pups, the growth trajectories of each animal from the two anesthesia exposure groups were extracted and compared using z-scores. Each rat was scanned four times during 4 weeks, and for each scan time point, the N-acetyl-aspartate (measured using proton magnetic resonance spectroscopy and LCModel analysis) was determined, and the corresponding z-score was extracted from the N-acetyl-aspartate growth curve, based on the animal age at the time of scan (fig. 2B). In both the single anesthesia–exposed animals (group 2) and multiple anesthesia–exposed (group 3), N-acetyl-aspartate increased over time regardless of the anesthesia exposure regimen. The N-acetyl-aspartate at 1 week of age was very similar across the two groups; however, both groups significantly deviated from the normal growth trajectory at 2 weeks of age (fig. 3). Further, at 1 week after the initial exposure(s),

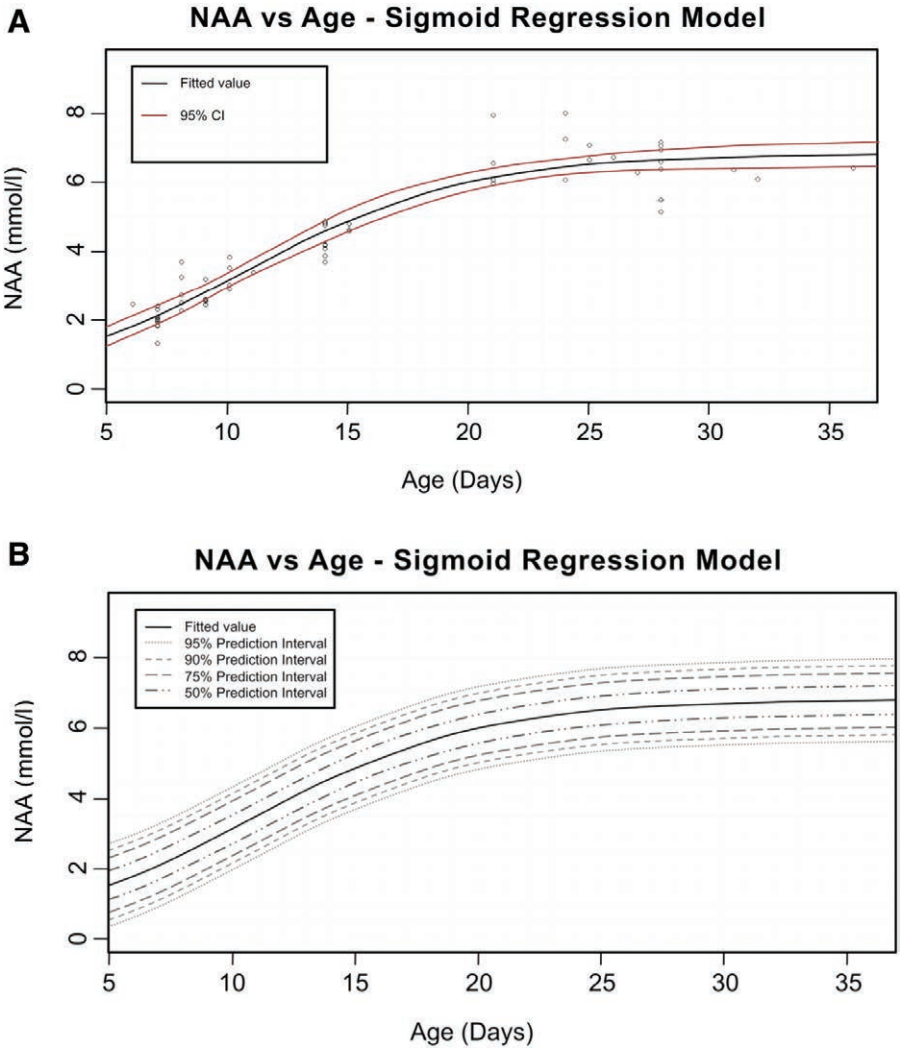

Fig. 2. Sigmoid regression model of N-acetyl-aspartate (NAA) plotted by rodent age over the first 4 weeks of life. This was derived from anesthesia naïve animals at ages ranging from postnatal day (PND) 5 to 35. (*A*) The fitted model displayed with the 95% CI lines has an R^2 value of 0.91 for this model. The open circles shown represent the NAA measurements from each of the animals tested. (*B*) The same sigmoidal regression model is here displayed with prediction intervals. The fitted value of this model was then further qualified by the 50, 75, 90, and 95% prediction intervals. These prediction intervals correlate to z-scores of ±0.5, ±1, ±1.5, and ±2, respectively. mmol = millimolar; R^2 = coefficient of determination.

group 3 demonstrated a more substantial deviation from the expected values, with a z-score (mean ± SD) of –1.87 ± 0.58 (P = 0.002), while the deviation from expected values in group 2 was less substantial, with a z-score of –0.80 ± 0.58 (P = 0.003). Furthermore, group 2 rat pups returned to normal N-acetyl-aspartate levels at 3 and 4 weeks, whereas group 3 animals did not demonstrate a complete return to baseline even at 4 weeks of age. In addition, a comparison looking only at group 2 and group 3 animals 1 week after exposure(s)—that is at 2 weeks of age—also demonstrated a significant difference (P = 0.010) between the two exposure groups.

Behavior

Novel Object Recognition Testing. Rats in all groups were given two 10-min sessions with the objects before being tested with the novel object. The animals did not exhibit any preference to either of the objects they encountered during these sessions; however, the groups showed significant differences when presented with a novel object. The multiple exposure animals (group 6) displayed a greater deviation from the controls (group 4) than the single exposure animals (group 5). The total time with the objects on day 2 was greatest for the group 4, and least for group 6 ($F_{(2,57)}$ = 4.65, P = 0.013) (fig. 4A). The number of approaches to the familiar object was not statistically different between the three groups; however, there was a stepwise difference in the number of approaches to the novel object, with the multiple-exposed group averaging the least approaches ($F_{(2,57)}$ = 3.66, P = 0.032) (fig. 4B). The primary outcome measure for this test, total time spent with the novel object, demonstrated the most significant

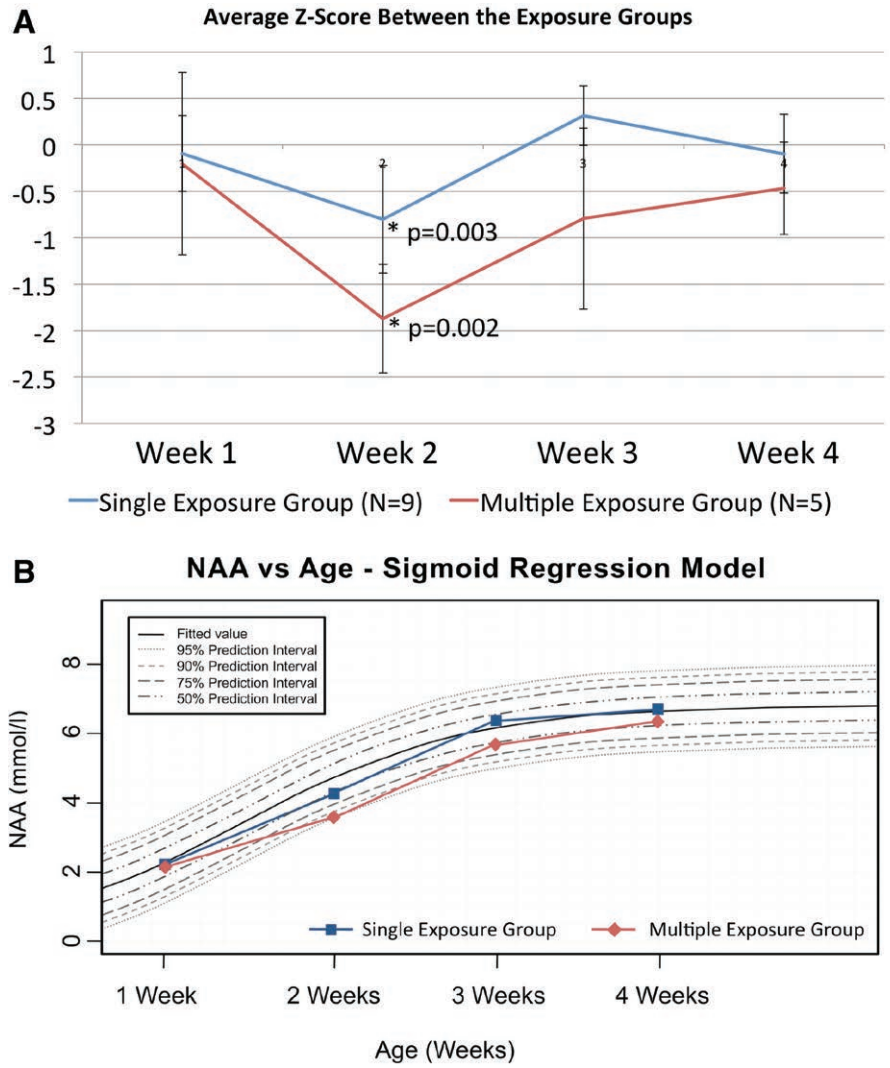

Fig. 3. A comparison of the N-acetyl-aspartate (NAA) levels of the two exposure groups to the anesthesia naïve animal group. Animals in the single exposure group were given sevoflurane anesthesia for 6 h on postnatal day (PND) 7, while those in the multiple exposure group were given sevoflurane anesthesia for 2 h on PNDs 5, 7, and 10, for a total of 6 h of sevoflurane anesthesia. (*A*) Comparing the average z-scores between the two groups, animals in both the single and multiple exposure groups were significantly different from the unexposed controls at 2 weeks old (1 week after the exposure[s]). The multiple exposure group was significantly different ($P = 0.01$) from the single exposure group. (*B*) The differences were plotted on the sigmoid regression model derived from the unexposed controls used as a "growth chart" for NAA. This demonstrates that, although the NAA for both exposure groups continued to increase, this increase was blunted in the single exposure group, and more severely blunted in the multiple exposure group. Furthermore, based on this model, NAA for the single exposure group returned to normal expected values by 3 and 4 weeks of age, while those for the multiple exposure groups remained lower than expected.

difference between the three groups, particularly when presented as the percentage of time spent as a function of the total time spent with both objects ($F_{(2,44)} = 4.65$, $P = 0.015$) (fig. 4C). Unexposed rats spent the most time with the novel object, and multiple-exposed rats spent the least. Figure 4D demonstrates the average positions of the animals in each of the three groups using group occupancy plots, showing that the unexposed animals spent a greater amount of time in the area around the novel object, while the multiple-exposed animals spent significantly less time with the novel object.

Open Field Testing. Testing in the open field demonstrated significant differences between the three groups, as shown in figure 5. These differences resembled those in the novel object recognition test, with the multiple exposure animals (group 6) displaying a greater deviation from the controls (group 4), compared to the single exposure animals (group 5). The total distance traveled during open field testing was significantly reduced ($F_{(2,57)} = 4.44$, $P = 0.016$) for group 6, compared to groups 4 and 5 (fig. 5A). The same was true of the total distance traveled in the outer zone ($F_{(2,57)} = 4.35$, $P = 0.018$). Another important

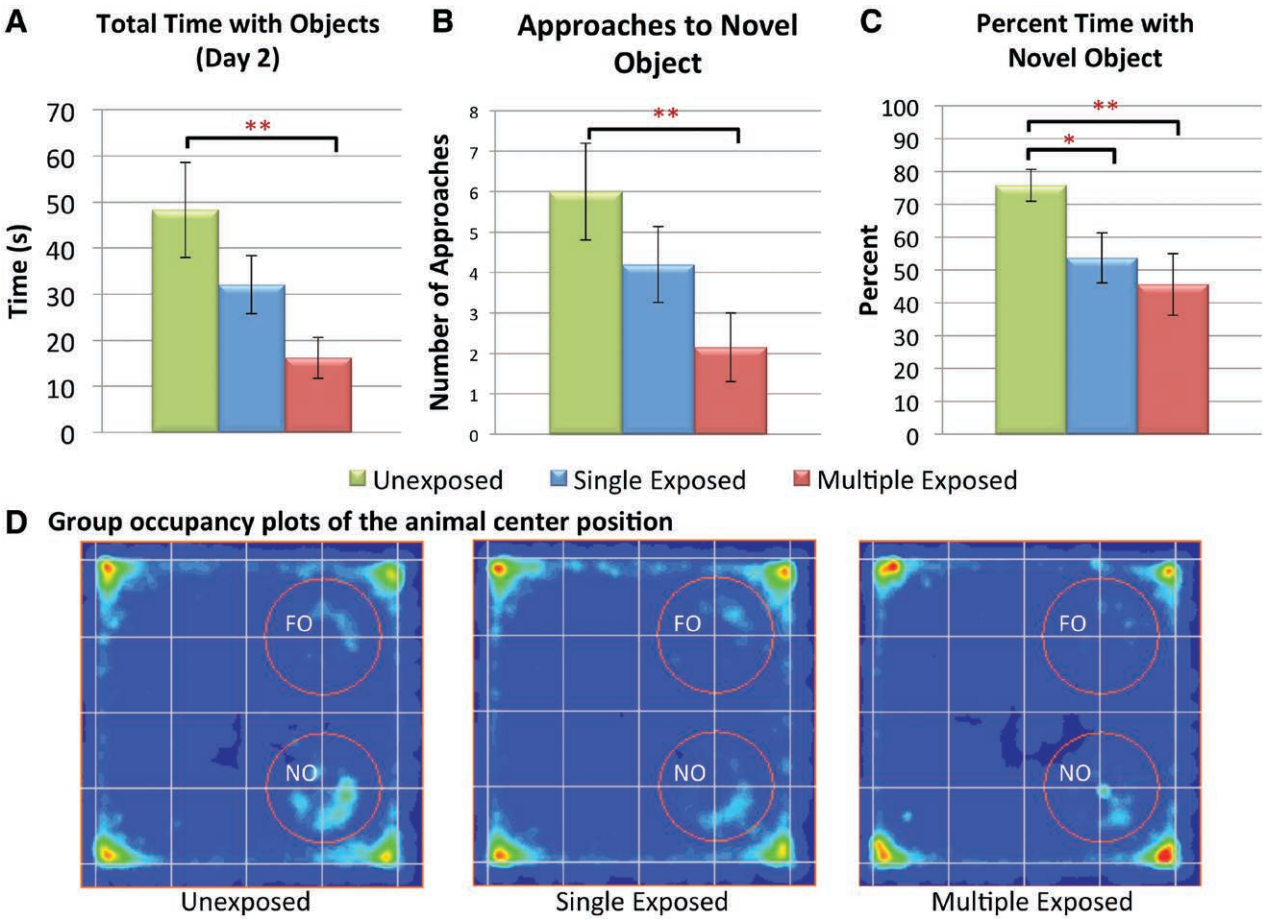

Fig. 4. Results of novel object recognition testing show that the multiple-exposed animal group displayed a greater deviation from the unexposed controls than the single-exposed group, as seen in the total time with objects (*A*), the number of approaches to the novel object (*B*), and especially with the percent time with the novel object (*C*). The group occupancy plot (*D*) shows the tracking of the center position for each group during testing. It can be seen that the unexposed controls spent the most time with the objects, with most of that time around the novel object. **P* < 0.05; ***P* < 0.01. FO = familiar object; NO = novel object.

difference between the experimental groups was the average distance away from the wall ($F_{(2,57)}$ = 3.87, *P* = 0.026), with multiple-exposed animals staying the closest (fig. 5B). Notably, the control group animals entered into the central zone more times ($F_{(2,57)}$ = 4.83, *P* = 0.012) than the other groups. Furthermore, as shown in figure 5C, control animals also traveled a significantly greater distance in that zone ($F_{(2,57)}$ = 4.13, *P* = 0.021), compared to those in the multiple exposure group (*P* = 0.008) or the single exposure group (*P* = 0.037).

Barnes Maze. We determined the average escape time on a daily basis for animals in each group during the training period, and found no significant differences ($F_{(2,57)}$ = 0.75, *P* = 0.479) in the learning patterns among the three animal groups (fig. 6A). All three groups exhibited a similar pattern of learning, with decreasing escape times after each trial, leading to a more direct and improved escape path from the start, improving further with each day of training. During probe trials, where the escape was removed from the Barnes maze, all groups exhibited a greater percentage of time spent in the target quadrant than would be expected by chance alone (data not shown). Figure 6B presents the occupancy plots for each of the three groups, tracking the position of the animals during probe trials. There was a trend for the animals in the unexposed group to spend a greater percentage of time in the target quadrant in the first minute of testing (64% compared to 50 and 56% for the multiple-exposed and single-exposed animals, respectively). This trend, however, was not statistically significant, and did not carry out through the full duration of the 2-min probe trial.

Home Cage Testing. As shown in figure 7, home cage testing did not reveal any significant differences between the three groups. Total number of beam breaks in both the dark and light phases was similar for the unexposed, single-exposed, and multiple-exposed animal groups. There was only one time period between time 00:00 to 00:30, where unexposed animals had significantly higher activity; however, this was not significant when corrected for multiple testing.

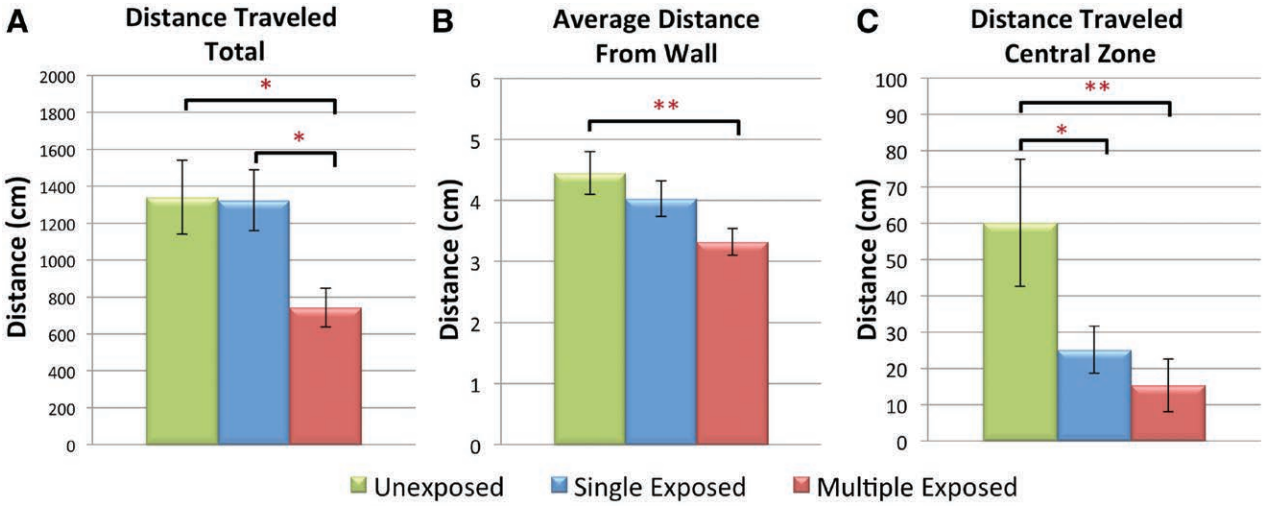

Fig. 5. Results of the open field rest demonstrate the effects of anesthesia exposure(s) on animal behavior. Unexposed controls were more active and traveled a greater distance during testing, compared to those with multiple exposures (*A*), and also were noted to ventures further from the walls of the open field (*B*). Moreover, both single-exposed and multiple-exposed groups spent less time and traveled less in the central zone (*C*), with the multiple-exposed group traveling the least distance in that zone. $^{*}P < 0.05$; $^{**}P < 0.01$.

Discussion

In light of clinical retrospective studies indicating that multiple anesthesia exposures may have a more detrimental effect on the developing brain than single exposures,[4,5] our objective was to compare the impact of these two anesthesia paradigms on brain development in a rodent model. Specifically, we examined the consequences of anesthesia exposure(s) on brain maturation (as determined by N-acetyl-aspartate growth trajectories), as well as on long-term memory and behavior conducted 3 to 5 weeks after exposure. We found that even though the total duration of anesthesia was identical for both exposure groups (3 × 2 h *vs.* 1 × 6 h), multiple short anesthesia exposures had a more detrimental effect on brain maturation, memory, and behavior than a single prolonged anesthesia exposure. These findings are important for several reasons. First, the animal behavioral data are in agreement with retrospective data in children demonstrating that the odds of developing a learning disability are greater for those children who have had two or more anesthesia exposures than for those with only one exposure.[4,5] Second, the introduction of the novel N-acetyl-aspartate growth chart approach will find utility beyond the current study. Third, we used the N-acetyl-aspartate chart to assess anesthesia toxicity in the developing brain noninvasively, an approach that can be used clinically in the future.

N-acetyl-aspartate, a known marker for neurons, increases in the developing brain, reflecting the increase in neuronal density and myelination during maturation.[10,28–30] We previously reported on the sensitivity of dynamically tracking N-acetyl-aspartate in the developing brain when assessing anesthesia-induced neurotoxicity, demonstrating that a single anesthesia exposure on postnatal day 7 can lead to a significant inhibition of the expected rise in the developing brain as early as 24 to 48 h after the exposure.[20]

The current study is the first to create and implement an N-acetyl-aspartate brain growth chart approach to track changes associated with neonatal anesthesia exposure(s), and to demonstrate use of this chart for assessing toxicity on a longitudinal basis. The basic concept of this growth chart is that N-acetyl-aspartate has a particular rate of increase during development, and therefore represents a unique maturation signature. This growth chart approach for evaluating normal brain development should be thought of as analogous to assessing an infant who does not gain appropriate weight, reflecting an underlying disease process. Similarly, a rat pup that does not demonstrate the appropriate increase of N-acetyl-aspartate in the brain should raise concern of possible abnormal brain maturation.

Our results demonstrate that all rat pups exhibited an N-acetyl-aspartate increase over the first 4 weeks of life, and that all the anesthesia-exposed animals showed a deviation from the expected normal growth trajectory at 2 weeks of age, only 1 week after the exposure(s). While the single-exposed animal group returned to baseline by 3 and 4 weeks, the multiple-exposed animals continued to have lower than expected N-acetyl-aspartate, suggesting impaired neuroplasticity. These anesthesia-induced changes in N-acetyl-aspartate may reflect persistent reduction in neuronal density,[16] impairment of neuronal myelination,[31] or may indicate persistent, long-term neuronal damage.[32,33] Intriguingly, the time of rapid N-acetyl-aspartate increase occurring between postnatal days 5 and 14 is highly correlated with the time of synaptogenesis and rapid neurodevelopment.[34–36] Importantly, this period correlates with the vulnerability period, during which

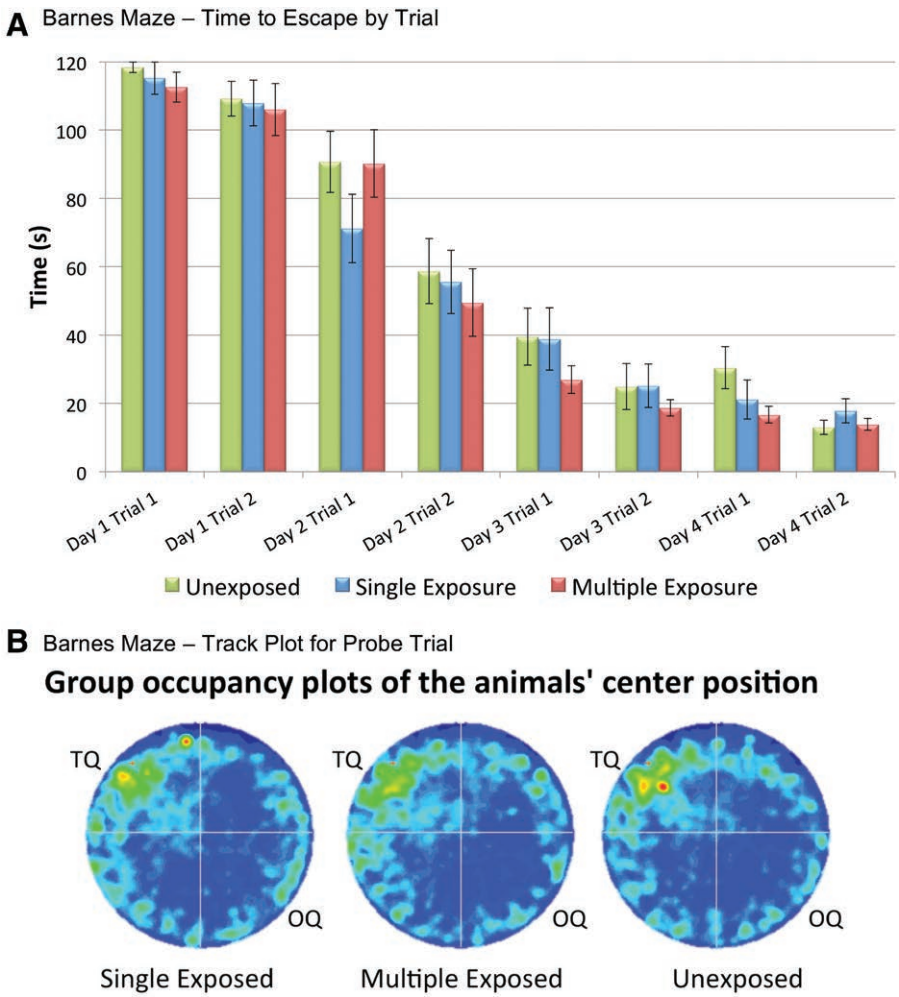

Fig. 6. The Barnes maze, a spatial memory recognition test, did not show significant differences between the controls and the anesthesia exposure groups. (*A*) The time to escape, shown by trail, showed a similar learning pattern for all three groups. (*B*) During the probe trial, where the escape was removed, the group occupancy plot shows a slightly higher intensity near the escape point during probe trials for the unexposed, but this was not significant. There was a trend for unexposed animals to spend more time around the target quadrant in the first minute; again, this was not significant and did not extend to entire duration of the probe trial. OQ = opposite quadrant; TQ = target quadrant.

anesthesia exposures are correlated with neurotoxicity and neuronal apoptosis.[3,37,38]

We also evaluated long-term effects of neonatal anesthesia exposure(s) on behavior and memory using a panel of tests. Evaluating episodic memory, we performed the novel object recognition test. Increased time spent with the novel object, as opposed to the familiar one, is thought to indicate that the animal remembers the familiar object. Importantly, the effect of multiple exposures was even more pronounced than that of the single long exposure, suggesting substantially impaired episodic memory.

Since the novel object recognition task takes place in an open field arena, it is important to establish that the pattern of novel object exploration was not the result of motor or temperamental changes in the animals. Here the open field test revealed a substantial qualitative difference in the effects of the two treatment regimens, where the animals subjected to multiple exposures demonstrated stronger tendencies for thigmotaxis (staying close to the walls of the open field) and avoidance of the central zone of the open field. Both of these behaviors may reflect a rise in anxiety-related behavior.[39] Alternatively, these changes may indicate overall locomotive and/or motor deficits.

The results in the novel object recognition and open field test need to be considered in the context of the lack of effect observed in the Barnes maze of either treatment condition. The Barnes maze requires intact locomotion and motor skills, as well as fear driven aversive motivation to enter the escape box. The lack of effect of either treatment groups is consistent with intact motor abilities and similar sensitivities to threat, arguing against enhanced anxiety-like behavior as a result of treatment. This lack of effect is initially puzzling in light of the apparent impairments in episodic memory found in the novel object recognition test. However, it should be noted that

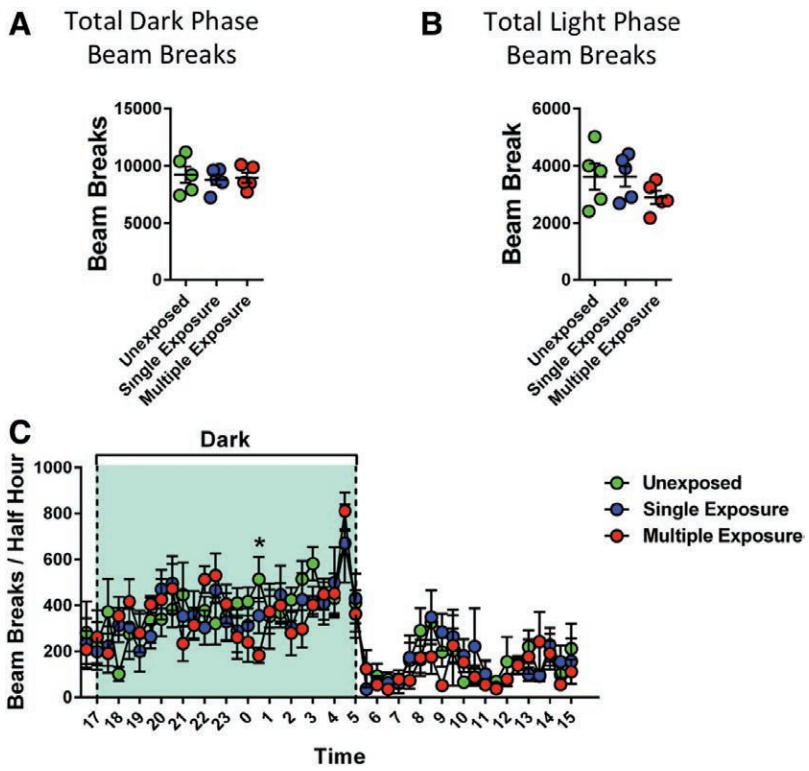

Fig. 7. Home cage testing performed in a subset of animals (n = 5 from each group) did not reveal any significant differences between the three groups, indicating that there was no motor or baseline behavioral deficits between the groups being tested. The data here is presented as (*A*) total beam breaks during the dark phase, (*B*) total beam breaks during the light phase, and (*C*) beam breaks per each half-hour of testing. Unexposed controls had higher activity during one time period between time 00:00 to 00:30, though not significant when corrected for multiple testing.

the fixed escape position version of the Barnes maze procedure presents the animal with a reference memory problem: learning the consistent, fixed location of the escape and associated extramaze cues. In contrast, the novel object recognition requires the ability to detect that a salient feature in an environment has changed. Therefore, the sparing of performance parameters in the Barnes maze may point to an important selective aspect of the memory impairments. Taken together, our data show that exposure to anesthesia during the vulnerability period results in substantial anxiety and behavioral consequences, and that multiple exposures elicit more profound behavioral changes than a single prolonged exposure.

Our results on the effect of anesthesia exposure on brain maturation and animal behavior are consistent with cytologic studies demonstrating a similar trend in individual cells, with multiple anesthetic exposures producing more significant ultrastructural damage to neurons in the developing brain than a single exposure, including more severe damage to synaptic and mitochondrial densities.[6] The higher severity of the damage seen after a second and third exposure is hypothesized to be the result of an exaggerated toxic response during reexposure after an initial insult.[6,7] There are a few ongoing prospective clinical trials looking into this problem in children, with preliminary results indicating that after one general anesthesia exposure, children evaluated at age 2 did not show any greater incidence of learning impairment.[40] There are multiple retrospective clinical studies that failed to find significant long-term deficits in children who have had only one anesthesia exposure during childhood,[4,5,41] suggesting that a single exposure may not have much clinically evident toxicity, whereas the toxicity resulting from multiple exposures may still have clinical manifestations.

There are several caveats in our study. First, it is not possible to have a proper control group of animals without anesthesia exposure for our proton magnetic resonance spectroscopy data, because of the fact that all spectroscopic data collection requires anesthetized animals. To partly circumvent this problem, we generated an "N-acetyl-aspartate growth curve" to help compare anesthesia-exposed animals to anesthesia-unexposed animals; this [calibrated] curve can now be used by future researchers in the field as a resource for evaluating the effects of different anesthetic agents and different exposure regimens. Though the fit for this curve was strong and predictive, deviation of the model from the data at particular time points may represent a misspecification of our model. Future studies should investigate other modeling approaches such as nonlinear mixed-effects models, or alternative methods, such as microwave fixation of the brain before proton magnetic resonance spectroscopy acquisition.[42,43] Second, our investigation did not directly study the hippocampus or cortical areas of the brain, but focused on the thalamus. Given that injury to either of

these regions leads to associated damage in the thalamus, and that memory function of the brain does not rely solely on the hippocampus, but rather on a complex network that includes the thalamus, we chose this region as a representative marker for cortical and hippocampal damage.[2,44–46] Finally, we focused on male animals to avoid potential complications introduced by the changing hormonal status in neonate and young adult females during exposure and testing; a detailed comparison of the N-acetyl-aspartate trajectories and response to single *versus* multiple anesthesia exposure between male and female animals remains an important challenge.

In conclusion, we demonstrated that N-acetyl-aspartate in the rodent thalamus continues to increase in the first month of life. We also showed that the normal increase in N-acetyl-aspartate is impeded by anesthesia, with multiple short exposures causing more deviations than a single exposure of equal length. While the N-acetyl-aspartate trajectory in single–exposed animals recovered to normal by 3 to 4 weeks of development, the multiple–exposed animals did not. Our studies also highlight the sensitivity and specificity of proton magnetic resonance spectroscopy for tracking of anesthesia-induced neurotoxicity by providing for longitudinal follow-up in the same animals (or in the future in humans). Furthermore, our results indicate that compared to single anesthesia exposure, multiple exposures produce a greater effect on animal learning, memory, and behavior when tested more than one month after the exposure. Future studies should focus on translating our preclinical data into the clinical domain.

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Competing Interest

The authors declare no competing interests.

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