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Brain Maturation in Neonatal Rodents is Impeded by Sevoflurane Anesthesia

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Abstract

Background—A wealth of data shows neuronal demise after general anesthesia in the very young rodent brain. Here we apply proton magnetic resonance spectroscopy (1HMRS), testing the hypothesis that neurotoxic exposure during peak synaptogenesis can be tracked via changes in neuronal metabolites.

Methods—1HMRS spectra was acquired in the brain (thalamus) of neonatal rat pups 24- and 48 h after sevoflurane exposure on post-natal day (PND) 7 and 15, and in unexposed, sham controls. A repeated measure ANOVA was performed to examine if changes in metabolites were different between exposed and unexposed groups. Sevoflurane-induced neurotoxicity on PND7 was confirmed by immunohistochemistry.

Results—In unexposed PND7 pups (N=21), concentration of NAA ([NAA]) increased by 16% from PND8 to PND9, whereas in exposed PND7 pups (N=19), [NAA] did not change and

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concentration of choline compounds ([GPC+PCh]) decreased by 25%. In PND15 rats, [NAA] increased from PND16 to PND17 for both the exposed (N=14) and unexposed (N=16) groups. Two-way ANOVA for PND7 pups demonstrated changes over time observed in [NAA] ($p=0.031$) and [GPC+PCh] ($p=0.024$) were different between those two groups.

Conclusions—We demonstrated that normal [NAA] increase from PND8 to PND9 was impeded in sevoflurane-exposed rats when exposed at PND7; however, not impeded when exposed on PND15. Furthermore, we showed that non-invasive $^1\text{HMRs}$ is sufficiently sensitive to detect subtle differences in developmental time trajectory of [NAA]. This is potentially clinically relevant since $^1\text{HMRs}$ can be applied across species, and may be useful in providing evidence of neurotoxicity in the human neonatal brain.

Introduction

The safety of anesthesia in children has recently become a topic of much scrutiny among anesthesiologists.^{1–3} The increasing concern is based on preclinical studies demonstrating increases in the number of cells undergoing apoptosis following anesthesia exposure(s) in the developing rodent brain, associated with long-term behavioral changes.^{4–9} Although this has also been seen in young, non-human primate brain,^{10–12} these important studies fall short of providing direct evidence of similar anesthesia-induced neurotoxicity in neonates and young children. So far, investigations on anesthesia-related neurotoxicity in humans have been limited to retrospective studies correlating the number of anesthesia exposures during early childhood with difficulties in learning and/or abnormal behavior and psychosocial issues.^{13–16}

A more direct approach to investigate whether anesthesia exposure(s) in the young human brain causes damage is by means of non-invasive imaging. Proton magnetic resonance spectroscopy ($^1\text{HMRs}$), which can be used to detect a variety of metabolites and biomarkers in the human brain as well as animal brain, might be useful for this purpose since it is non-invasive. Small molecular weight moieties such as N-Acetyl-Aspartate (NAA, neuronal marker), myo-inositol (myo-Ins, marker of glia cells and inflammation), and choline compounds (marker of cell membrane turnover), in addition to metabolites involved in energetics (i.e. glutamate, glucose and lactate), can be quantified by non-invasive $^1\text{HMRs}$.^{17–20} For example, in clinical trials of patients with Alzheimer's disease (AD), NAA is used as a biomarker to track disease progression; total gray-matter NAA has been shown to decline (reflecting neuronal loss) in AD patients compared to controls and/or non-treated AD subjects.^{21–24} NAA has also been identified as a potential biomarker for traumatic brain injury (TBI), specifically for mild TBI, which is not always associated with obvious structural changes.²⁵ We recently demonstrated feasibility of using *in vivo* $^1\text{HMRs}$ to characterize metabolic profiles in the brain of children during anesthesia, identifying different cerebral metabolic status with inhalational compared to propofol anesthesia.²⁶ $^1\text{HMRs}$ can also be used to track normal brain maturation in the very young brain via changes in NAA.^{27–31} Specifically, NAA has been shown to increase from 31–45 weeks of gestation³² and this continues into the first decade of life in children, reflecting normal human brain development.^{32–34} Similarly, in rodents, NAA, myo-inositol and choline compounds are among the most sensitive markers for tracking brain maturation.²⁷

NAA is also an important biomarker for brain neurotoxicity. For example, abnormal (lower) levels of NAA have been demonstrated in children undergoing treatment for acute lymphoblastic leukemia,³⁵ and/or inborn errors of metabolism.^{36,37} The striking evidence of neurotoxicity in neonatal rodents after anesthesia exposure(s) is highly likely to be reflected as derangements of the metabolic profiles including NAA, myo-Ins or choline. Thus, based on previous reports, we hypothesized that neurotoxic exposure to inhalational anesthesia of neonatal rats, when the brain is most vulnerable to anesthesia toxicity during peak synaptogenesis, would be reflected in changes of metabolic profiles, in particular NAA obtained *in vivo* by ¹HMRS. We further hypothesized that the same exposure scheme would not lead to such changes in older less vulnerable rats.

Materials and methods

Animals

The local institutional animal care and use committees at Stony Brook University, Stony Brook, NY, USA and Brookhaven National Laboratory, Upton, NY, USA approved all animal work. Sprague Dawley® (SD) male rats were used for the study. Lactating dams-with-litter were ordered using only male pups (Taconic, NY, USA). Table 1 shows the different experimental groups. At the appropriate ages, the rat pups were randomly divided into four groups based on age and anesthesia exposure regimen. Groups 1 and 3 were exposed to 1 MAC sevoflurane for 5 h on post-natal day (PND) 7 and PND 15, respectively (designated 'exposed' groups). Groups 2 and 4 served as sham-controls and not anesthetized on PND 7 and 15, respectively (designated 'unexposed' groups) (Table 1). All rats were weighed daily over the course of the study.

Animal Preparation

Exposed Groups: Group 1 and 3 rats were separated from their mother on PND7 and PND15, respectively. Anesthesia was induced with 5% sevoflurane in 100% oxygen in an induction chamber and when the righting reflex was gone, anesthesia was maintained at 2.2% sevoflurane delivered in a 1:1 O₂:Air mixture. During anesthesia, the animals were placed on a warming pad and their body temperature strictly controlled to 37 ± 1°C, while also monitoring heart rate, respiratory rate, and oxygen saturation. The total duration of exposure to sevoflurane for each animal was 5 h. Minor adjustments were made to the anesthetic inhalational regimen as needed to maintain an average respiratory rate between 60–70 breath per minutes and average heart rate between 300–400 beats per minutes. After the 5 h anesthesia exposure, the rats were allowed to recover (recovery = regaining of righting reflex). Upon recovery, the rats were returned to their mother. *Unexposed Groups:* Group 2 and 4 rats were removed from their mother on PND7 and PND15, respectively, and observed in the induction chamber for the duration of the 5 h but without anesthesia and physiological monitoring; however, skin temperature was checked periodically to be certain that the animals were maintaining normal body temperature. After completion of the 5 h period, unexposed control rats were returned to their mother. Experimenters were not blinded to animal groups.

¹H MRS and anesthesia

¹H MRS spectra was acquired on a 9.4 Tesla MRI instrument interfaced to a Bruker Advance console and controlled by Paravision 5.0 software (Bruker Bio Spin Corp, MA, USA). A custom-built animal cradle system was used for accurate and consistent positioning of the animal head in relation to the surface coil and the center of the magnetic field. Each rat in groups 1 and 2 was scanned twice, on PND8 and PND9; and each rat in groups 3 and 4 was scanned on PND16 and PND17 (Table 1). For ¹H MRS scanning, the rats were first induced with 5% sevoflurane, and then maintained with 2.2% sevoflurane mixed in a 1:1 O₂:Air mixture while continuously monitoring their heart rate, respiratory rate, body temperature, and oxygen saturation using optical monitors compatible with MRI (SA Instruments, Inc., Stony Brook, NY). Body temperature was strictly controlled using a computer assisted heating system (SA Instruments, Inc., Stony Brook, NY) to maintain temperature at 37 ± 0.5°C during acquisition times. For radio-frequency (RF) signal transmission and reception, an 11.2 cm RF volume coil and a custom-built 3-cm surface RF coil, respectively, were used. After positioning in the center of the magnet, anatomical images of the brain were acquired in three orthogonal planes using a RARE sequence (TR=2500, TE=40ms, NA=2, RARE factor=8, number of slices=25, in plane resolution = 0.117mm/pixel, slice-thickness=0.9mm, slice gap=0.1mm) in order to identify the location of the thalamic region. A single voxel of 3mm × 2mm × 3mm was placed covering the thalamic region of both hemispheres (Fig. 1A). B₀ inhomogeneity in the voxel was minimized by applying both first and second order shims using MAPSHIM. ¹H MRS was acquired using point resolved spectroscopy sequence (PRESS) (TR=4000ms, TE=12ms, NA=1024, spectral width=8012 Hz, number of acquired complex points=4096; scan time ~69 minutes). Each free induction decay (FID) signal was recorded separately and corrected for frequency and phase changes during the scan using custom software written in MATLAB® (The MathWorks, Inc., MA, USA). A typical ¹H MRS spectrum from thalamus of a PND8 rat pup is shown in Fig. 1B. For the quantification of metabolite concentrations, a water unsuppressed scan was acquired using the same MR parameters as used for the water suppressed scan. Once completed, the animals were removed from the scanner and allowed to recover from the anesthetic before being returned to their litter. This process was repeated for each animal the next day (Table 1).

Spectral processing

Phase and frequency coherent summed spectra were analyzed using LCModel software (Stephen Provencher Inc., Ontario, Canada)³⁸ and fitted by using a set of 18 simulated metabolites: alanine (Ala), aspartate (Asp), creatine (Cr), phosphocreatine (PCr), γ-aminobutyric acid (GABA), glucose (Glc), glutamine (Gln), glutamate (Glu), glycerophosphorylcholine (GPC), phosphorylcholine (PCh), glutathione (GSH), myo-inositol (myo-Ins), scyllo-inositol (scyllo), Lactate (Lac), N-acetylaspartate (NAA), N-acetylaspartylglutamate (NAAG), phosphoethanolamine (PE) and taurine (Tau). In addition, simulated spectra of mobile lipids (Lip20, Lip1.3a, Lip1.3b and Lip09) were also included. Spectral profiles of population averaged macromolecules (MM) from a separate group of juvenile rats³⁹ were also incorporated as described previously⁴⁰. Due to the higher concentration of water in neonatal rat brains²⁷, the quantitative data was linearly

interpolated and the water concentrations were estimated as 48.6 mM for PND8, 48.4 for PND9, 46.7 for PND16, and 46.5 for PND17.

The paired ^1H MRS spectra obtained over two consecutive days from each animal were carefully examined following processing and LCModel analysis in order to assure that they matched in quality. Matching of spectral quality was assured by comparing signal-to-noise ratio (SNR) and full width half maximum (FWHM) derived from LCModel analysis from the two spectra. Paired spectra from rats where the SNR and/or FWHM deviated more than '4' and/or 0.020ppm, respectively, were excluded from analysis. In addition, we excluded spectra with obvious motion artifacts and aberrant baseline fitting. The statistical analysis focused on comparing changes of five key metabolites (NAA, (GPC+PCh), Glu, myo-Ins and Cr+PCr) in exposed and unexposed rats. The Cramer-Rao lower bounds (CRLB), which estimates the accuracy of the fitted concentrations, were below 15–20% for most of these metabolites.

Statistical analysis

Statistical analyses were performed using SAS version 9.3 (SAS Institute Inc., NC, USA) and XLSTAT Version 2011 (Addinsoft, NY, USA). Normality was examined using the Shapiro-Wilk test. A two-way repeated measures ANOVA was performed to examine if the pre-selected metabolite concentration changes from PND8 to PND9 were different between groups 1 and 2. For all pre-selected quantified metabolites, a two-sided paired t-test was performed as post-hoc testing to examine how these metabolites changed between day 8 and day 9 within each group, respectively. Similar analysis was also applied to group 3 and 4 rats. All data are presented as mean $\pm$ standard deviation. We executed a preliminary power analysis based on ^1H MRS in vivo data demonstrating that [NAA] in normal neonatal brain increases 0.5mM/day from PND7 to PND10²⁷, and the hypothesis that brain growth reflected by [NAA] increases in sevoflurane-exposed neonatal rat brain would be reduced to 0.1 mM or less/day. According to this preliminary data analysis, the assumption was made that the NAA change from PND8 to PND9 would be from 2mM to 2.1mM for the sevoflurane-exposed group 1 rats, and 2mM to 2.5mM for the unexposed group 2 rats. The pooled NAA standard deviation for the two groups is assumed to be 0.25, and the standard deviation of NAA change is assumed to be 0.4. Data generated with R function 'mvrnorm' (package 'MASS') for 1000 times, and 'aov' was applied to calculate the p-value of group-by-time interaction effect for each simulation. Percentage of simulations with a p-value of interaction effect less than the significance level 0.05 is regarded as the power. A total sample size of 46 (23 for each group) was found to achieve a meaningful power of 0.85 to detect a significant group by time interaction effect.

Histology

A subset of PND7 rats was processed for histology 1 h after exposure to sevoflurane or non-exposure. The rats selected for histological processing underwent trans-cardiac perfusion fixation accomplished by placing the animal under very deep anesthesia (5% sevoflurane). The left ventricle was cannulated with a 23 gauge blunt-tipped needle (Intramedic® Luer Stub Adapter; Becton, Dickinson and Company, NJ, USA) and directed towards the base of the aorta. Following cannulation, the right atrium was slit open to allow for venting, while

1ml/g of heparinized saline was slowly injected, immediately followed by a slow injection of 1ml/g of 4% formaldehyde solution. Upon completion, the brain was extracted, placed in 4% formaldehyde solution for 24 h, and then placed in phosphate buffer solution [PBS] until processing.

Immunohistochemistry

The harvested brains were processed in the following manner; first the left hemisphere of the brain was sectioned at 80 μ m in the sagittal plane using a Vibratome ® 1500 (Leica Microsystems Inc., IL, USA). Brain sections were serially collected and sequentially assigned every sixth section, at 480 μ m apart. The brain sections were stored at 4°C in phosphate buffered saline (PBS) with 0.1% sodium azide. For immunohistochemistry, caspase 3 immunohistochemistry was performed as previously described⁴¹. Briefly, this was done by first washing the sections with PBS, then incubating the samples in PBS with 5% goat serum and 1% Triton X-100 for 2 h at room temperature. The sections were then washed and incubated overnight at 4°C and additionally for another 2 h at room temperature, with the primary antibodies mixed with PBS, 3% goat serum, and 0.2% Triton X-100. The primary antibodies were rabbit anti-activated caspase 3 (Cell Signaling, MA, USA, #9661S, D175; 1:400 dilution) for degenerating cells, mouse anti-NeuN (Millipore, MA, USA; MAB-377; 1:800 dilution) for neurons, and Hoechst33342 (Life Technologies, NY, USA; 1 μ g/ml), a nucleus counter-staining dye. The sections were then incubated for 2 h for the secondary antibody reaction in PBS, 3% goat serum, and 0.2% Triton X-100. Secondary fluorescent dye-conjugated antibodies (Life Technologies, 1:400 dilution) were goat anti-rabbit IgG-Alexa Fluor- 488 (AF-488) for anti-caspase 3 and goat anti-mouse IgG-AF-568 for anti-NeuN. The sections were then washed and mounted onto slides using fluorescent mounting medium DakoCytomation (Agilent Technologies, Denmark) and stored in 4°C until imaging. The microscope used for image acquisitions was a Zeiss LSM780 confocal laser scanning microscope with a fully automated stage for image tiling, multiple laser lines, and advanced spectral separation (Carl Zeiss Microscopy GmbH, Germany). Images were acquired at both 5 $\times$ magnification for whole brain imaging and at 20 $\times$ specifically for the thalamus. Representative image of caspase positive neurons with nuclear changes was taken under 63 $\times$ magnification. Zen Image Analysis Software (Carl Zeiss Microscopy GmbH, Germany) was used for the creation of stitched whole brain images as well as stitched images of the thalamus. Image brightness and contrast were enhanced using Apple Aperture software version 3.4.5 (Apple, CA, USA).

Results

The rats in groups 1 and 2 demonstrated significant weight gain, increasing by ~20% from PND7 to PND9, as would be expected during this neonatal period (Fig. 2A). A two-way repeated measures ANOVA was executed to examine the differences in weight gain between the two groups, and no significant time*group effect was found. Groups 3 and 4 rats also demonstrated an expected increase in weight from PND15 to PND17 (Fig. 2B), and again, no significant time*group effect was found between exposed and unexposed groups.

Quantitative analysis of metabolites

After excluding scans with poor quality as determined by a full width half maximum [FWHM] of 0.040 or greater, a signal-to-noise ratio [SNR] of 10 or less, and/or scans with poor baselines, ¹HMRS spectra from a total of 40 PND7 rats (exposed rats - Group 1 (N=19); unexposed rats - Group 2 (N=21)) were of sufficient quality to be included in the final data analysis. Table 2 shows the concentrations of the five metabolites measured on PND8 and PND9 and demonstrates that the concentration of NAA ([NAA]) increases by 16% from 2.08mM to 2.41 mM (p-value < 0.0001) in the unexposed group 2 rats; however, this increase is not evident in the sevoflurane exposed group 1 rats over the same developmental time period. In addition, the concentration of choline compounds appears to decrease by 25% in the exposed rats from PND8 to PND9; but is unchanged in the unexposed, control rats. Figure 3 illustrates these profile differences specifically for [NAA] (Fig. 3A) and [GPC+PCh] (Fig. 3B); as can be seen, the rate of rise in [NAA] is less in the exposed rats compared to the unexposed rats. Thalamic [NAA] and [GPC+PCh] is therefore reduced in the PND9 animals which were previously exposed to sevoflurane. Table 2 also shows the result of the ANOVA analysis demonstrating the changes over time observed in [NAA] (p=0.031) and [GPC+PCh] (p=0.024) from PND8 to PND9 were different between groups 1 and 2, with a significant interaction effect by group and time.

For the older group of animals, ¹HMRS spectra from a total of 30 animals (exposed rats Group 3 (N=16); unexposed rats Group 4 (N=14)) were of sufficient quality to be included in the final data analysis. P-values of group-by-time interaction effect from the two-way repeated measures ANOVA in Table 3 shows that all of the metabolite concentration changes from PND16 and PND17 were not different between the two groups. In other words, the increases from PND16 to PND17 were the same in both groups and not affected by anesthesia exposures as shown for the neonatal rats. As is shown in Figure 3, the concentration of [NAA] increased by 16% from 4.45mM to 5.00mM (p-value < 0.01) in the exposed rats from group 3, and [NAA] increased by 9% from 4.58mM to 4.97mM (p-value < 0.01) in the unexposed rats from group 4 (Fig. 3C). The [NAA] concentration change between these two groups was not different (p=0.436). The observed change in [GPC+PCh] from PND16 to PND17 (Fig. 3D) also demonstrates no difference between these two groups (p-value=0.674).

Since previous studies have demonstrated a possible association of mobile lipids with apoptosis,^{42–44} neuronal loss, and/or neuronal stem cell generation,^{45,46} we were also interested in exploring whether there were changes in mobile lipids resonating at ≈1.30ppm and/or 0.9ppm in the sevoflurane-exposed rat pups. The spectral signature at 1.30ppm (represented by [Lip13a]) showed that there was a trend towards increase in the exposed group, particularly on PND8; however, this difference was not statistically significant (p>0.05, results not shown). The LCModel derived [Lip13a] in the spectra was associated with large errors (as measured by the CRLB), probably caused either by very low tissue concentrations of mobile lipids and/or insufficient spectral resolution and SNR. To increase SNR we summed spectra obtained from each group on PND8 and PND9. When compared in this manner, [Lip13a] was found to be higher in the sevoflurane exposed animal group on PND8 seen in Figure 4A. Furthermore, Figure 4B demonstrates that there was no difference

in summed [Lip13a] for the older rats between exposed and unexposed on PND16. Although these findings corroborate the hypothesis that mobile lipids are higher in the PND7 sevoflurane-exposed rat pups, this result needs to be interpreted very carefully, because reliability of lipid peaks are influenced by broad and overlapping neighboring peaks of macro-molecules (i.e. MM02).

Histology

In a subset of PND7 rats from each of the two groups, the brains were harvested 1h after exposure or non-exposure and processed for histological evidence of caspase 3 positive cells, indicating cells undergoing apoptosis. Figure 5 shows representative sagittal sections of whole brain images from each group with a clear difference in the number of caspase 3 positive cells (green-colored cells) throughout the brain exposed to sevoflurane for 5 h on PND7 (Fig. 5B) in comparison to the unexposed brain (Fig. 5A). Figure 6 demonstrates higher-powered views of the thalamic region from an unexposed rat (Fig. 6B) and a rat exposed to 5 h of sevoflurane on PND7 (Fig. 6C – E), confirming a larger number of apoptotic cells in the anesthesia-exposed animals (compare Fig. 6B with Figs. 6C – E).

Discussion

We demonstrated differences in the rate of rise of [NAA] from PND8 to PND9 in neonatal rats exposed to 5 h of sevoflurane anesthesia on PND7 when compared to age-matched unexposed rat pups. Furthermore, we showed that the rate of rise of [NAA] from PND16 to PND17 was not affected by the same anesthesia regimen compared to sham controls. These metabolomics findings are important because it supports histological data demonstrating PND7 rats exposed to anesthesia undergo increased apoptosis; whereas older rats (>PND14) are not as sensitive. The increased vulnerability of neonatal PND7 rats to anesthesia-induced neurotoxicity has been correlated to the time of peak of synaptogenesis.^{8,47} Because NAA is a marker of neuronal integrity and metabolism,⁴⁸ and [NAA] increases reflect normal brain maturation in rodents²⁷ as well as in humans^{49–51}, we interpreted our findings as indirect evidence of sevoflurane-induced neurotoxicity. This assumption was strongly supported by the demonstration of 1) parallel decreases in choline (likely reflecting less ‘new’ cells), 2) demonstration of more apoptotic cells in the thalamus of sevoflurane-exposed rat pups when compared to age-matched unexposed rats, and 3) lack of a similar change in [NAA] in the older animal exposure group who are not susceptible to the neurotoxic effects of anesthesia.

The main goal of this study was to characterize changes in key metabolites of rat pups from PND8 to PND9 as measured by ¹HMRS. Baseline [NAA] at PND8 was ~2.1mM; and in unexposed, control rat pups [NAA] increased significantly by 16% over 24 h which is in agreement with previous reports.²⁷ Specifically, Tkac et al. showed that [NAA] increases from 2mM to 3.8mM from PND7 to PND10, or approximately 0.5mM/day.²⁷ Since NAA is a known marker for neuronal integrity and metabolism, the NAA increase is inferred to be the result of an increasing number and size of neurons and myelination in the brain during early stages of development. The expected brain growth reflected as an incremental [NAA] increase was not observed in the sevoflurane-exposed rat pups (Fig. 3A). The documented differences in the daily rate of rise of [NAA] between the two groups of neonatal rat pups,

although small, might represent a real, quantifiable loss of neurons as a result of anesthetic exposure at this age. The fact that an altered [NAA] trajectory was observed only in the PND7 rats (a post-natal time point highly susceptible to neurotoxic effects of anesthesia), and was not observed in the PND15 rats (known to be less susceptible) further supports the claim that this may in fact represent a quantifiable loss of neurons in the PND7 neonatal rats. This possibility is also supported by other published reports. First, NAA is located and synthesized in neurons,⁵² and [NAA] increases during early post-natal brain development and is involved in myelin synthesis.⁴⁸ Second, there are several recent ¹HMRS studies in human demonstrating that low [NAA] is indicative of neonatal brain damage.⁵³ For example, in a recent study cerebral [NAA] was measured in infants (gestational age 39 ± 1.8 weeks) with mild or severe encephalopathy and compared with age-matched normal infants and found to be 20–50% lower in the infants with encephalopathy.⁵³ Third, ¹HMRS has been applied to document changes after mild traumatic brain injury (mTBI) in military personnel, as well as professional and semi-professional athletes; there is often no evidence of direct parenchymal brain injury,^{54,55} but specimen and post-mortem studies have demonstrated apoptosis in human brains afflicted with mTBI.⁵⁶ For example, in a recent study looking at athletes who had suffered concussions, [NAA] ratios were found to decrease.⁵⁷ Although we documented neuronal apoptosis in the sevoflurane-exposed rats, we did not observe a decrease in [NAA] as is observed in mTBI.⁵⁷ The main difference is that the [NAA] in the developing brain is constantly increasing, which is not the case in the adult brain. Therefore, neuronal injury via NAA would more likely be reflected as a change in the trajectory of [NAA] rather than a decrease. Nevertheless, it is important to point out that [NAA] decreases in association with brain injury, such as stroke or TBI, can be transient and several studies have reported near-complete recovery of NAA levels after ischemia.^{25,48} In other words, the [NAA] decreases observed from PND8 to PND9 in sevoflurane-exposed rats may be recoverable at later post-natal stages. The key question remains, however, whether this change in [NAA] is indicative of any long-term cognitive deficits. Future studies focused on implementing long-term follow up and behavioral testing will answer this question.

The fraction of neurons that undergo apoptosis as result of anesthesia exposure at this critical time point in development, albeit much higher compared to normal, is still relatively small when considering the total number of neurons in the brain. This can explain why a large sample size was needed to have sufficient power to detect a significant interaction effect by our repeated measures ANOVA analysis. This may also give some credence to the theory that the damage caused by anesthesia may not be long lasting, and potentially reversible, especially given the plasticity of the brain at that age. On the other hand, the fact that we can measure a change in [NAA] given this small fraction of injured neurons may point more towards the opposite conclusion, that there are some underlying metabolic changes taking place that can have some long lasting, deleterious effects. For this to be better understood, more follow-up testing would be needed to look at changes in [NAA] over the complete developmental time-course in exposed animals. Furthermore, there is evidence demonstrating that the neurotoxic effects of anesthetics in neonatal rat pups can be decreased by inhibition of the NKCC1 pathway using bumetanide, as well as by certain anesthetics, such as dexmetomidine.^{58–61} Further studies would be needed to determine if

bumetanide, dexmetomidine, or other agents that reduce anesthesia neurotoxicity might annihilate the observed changes in the [NAA] trajectory resulting from the anesthesia exposure in neonatal PND7 rat pups.

The decrease in the total choline as measured from PND8 to PND9 in the exposed animal group is also noteworthy. The total choline measured by ¹HMRS in our study mainly represents phosphocholine and glycerophosphocholine; and these compounds become mobile (and measurable by ¹HMRS) when the cell membrane has broken down. A change in choline is interpreted as a change in cell membrane turn over. For example, an increase in total choline is observed in certain neoplastic lesions.^{62–63} The decrease in choline observed from PND8 to PND9 could therefore signify impeded brain growth within the region investigated which may have long-term sequelae for cognitive and behavioral development. Further studies characterizing the association between long-term memory impairment and choline deficits during neuronal development would be needed in order to corroborate such possibilities.

There are several limitations inherent to this study. First, acquiring ¹HMRS spectra from neonatal rat pups requires exposure to general anesthesia; therefore, we do not have a complete ‘non-anesthesia’ control of NAA and other metabolite changes from PND8 to PND9. Given that the preponderance of evidence that any anesthesia exposure or multiple exposures during the vulnerability period can cause damage to neurons as well as cognitive impairment, it is crucial to point out this drawback to the study. It is possible that a larger increment would have been observed in the controls if the studies could be done in an awake setting. There is very limited evidence of ¹HMRS metabolites measured in awake animals, given the technique’s sensitivity to motion and the ultimately stressful impact of restraint during long scanning. Some of these technical difficulties might be overcome with appropriate, comfortable, sound-isolated animal holders, particularly given that MRI can be accomplished in neonatal and young human infants without anesthesia.^{64–66} Another limitation is the short post-natal developmental period studied and the lack of long-term behavioral data to elucidate the impact of the observed [NAA] changes. We are in the process of developing the means necessary to execute such studies.

In conclusion, our data shows that non-invasive ¹HMRS is sufficiently sensitive to detect subtle differences in the developmental time trajectory of [NAA] in the neonatal rodent brain. This is potentially clinically relevant since ¹HMRS can be applied across species and therefore a similar approach can be used to provide more direct evidence of neurotoxicity in the human neonatal and/or young brain from anesthesia exposure(s). Normal time trajectories of NAA changes during development are well documented in human brain^{29,67,68} and could be used in future studies focused on assessing human brain injury related to anesthesia exposures.

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Figure 1.

Representative images shown here show the area of focus for ^1H MRS scanning. (A) ^1H MRS scans were acquired from the thalamic region of both hemispheres, allowing for a large enough voxel size to have acceptable SNR and spectral resolution. Typical positioning of the voxel is shown here in three different orthogonal planes acquired by RARE sequencing. (B) After acquiring 1024 averages over the course of ~1 hour, a representative spectrum is shown. The highest peaks for this animal age group tend to be total choline (tCho) and total creatine (tCr); whereas N-Acetylaspartate (NAA), which is typically the highest peak in

older animals, tends to be lower in PND7 rat pups. (^1H MRS = proton magnetic resonance spectroscopy, Cb = cerebellum, GABA = gamma-Aminobutyric acid, Glu = glutamate, Glx = glutamate and glutamine, ml = myo-inositol, MM = macro molecules, NAA = N-Acetyl Aspartate, Olfac = olfactory bulb, PND = post natal day, ppm = parts per million, SNR = signal to noise ratio, Tau = Taurine, tCho = total choline, tCr = total creatine)

Figure 2.

The plot of animal weight in grams by group average demonstrates that all animals gained weight appropriately during the course of the experiments in both exposed and unexposed groups for the (A) PND7 and (B) PND 15 animals, as would be expected during this developmental phase. (*PND = post natal day*)

Figure 3.

(A) [NAA], which is a marker for neurons, demonstrated significant increases for the PND7 unexposed animal group ($p < 0.0001$); however, [NAA] remained relatively unchanged in the PND7 exposed group, indicating possible neuronal damage or impaired neuronal growth during that period. (B) The exposed PND7 rats demonstrated a decrease in total choline ($p < 0.02$), while in the unexposed PND7 rats had a slight increase. (C) The older PND15 rats demonstrated an increase in [NAA] irrespective of anesthesia exposure, identifying that the decrease [NAA] seen in PND7 rats is unique to that age group. (D) Similarly, the PND15

exposed and unexposed rat groups both demonstrated similar trends with total choline levels, again demonstrating that the difference seen in response to anesthesia exposure on PND7 is unique to that age group. (*GPC+PCh* = *total choline*, *NAA* = *N-Acetyl Aspartate*, *PND* = *post natal day*)

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Figure 4.

Summed spectra for both the unexposed and exposed animal groups, shown here in the top portion, can be used to increase the signal to noise ratio, allowing for better comparison of metabolites that have poor spectral resolution, such as Lip13a. The bottom portion of this graph depicts the results of isolating for the Lip13a peak. (A) The PND7 exposed rats demonstrate a higher peak on PND8 as compared to the unexposed group. (B) For the PND15 anesthesia exposed and unexposed groups, there is no difference in the height of the Lip13a peaks when measured on PND16. The scale for all Lip13a isolated peaks was doubled for visual effect; however, it is clear that there is a slight overall increase in the Lip13a signal for the PND7 exposed group. Broad and nearby macromolecule peaks can highly influence this summed spectra, and careful interpretation is cautioned. (*GABA* = gamma-Aminobutyric acid, *Glu* = glutamate, *Glx* = glutamate and glutamine, *Lip13a* = Lipid peak at 1.3 parts per million, *mI* = myo-inositol, *MM* = macro molecules, *NAA* = N-Acetyl Aspartate, *PND* = post natal day, *ppm* = parts per million, *Tau* = Taurine, *tCho* = total choline, *tCr* = total creatine)

Figure 5.

Histological images of a lateral section of the rat brain in the sagittal plane, taken from an unexposed PND7 rat pup (A), and from a PND7 rat pup after 5 hours of sevoflurane anesthesia exposure (B). These representative images were taken at 20× magnification using laser fluorescent microscopy to detect **caspase3** depicted in green, staining for apoptotic cells, as well as both NeuN and Hoechst33342 depicted in grayscale, staining for neurons and nuclei, respectively. The exposed animal brain has an increased number of cells that are caspase3 positive throughout the brain. Inserts depict a closer view of the cortex with

caspase3 depicted in green, **NeuN** depicted in red, and **Hoechst33342** depicted in blue; the increase in number of apoptotic cells is clearly seen, particularly in the internal pyramidal and multiform layers of the cerebral cortex.

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Figure 6.

Stitched images were taken of the thalamic area as depicted (A) using 20× magnification from an unexposed PND7 rat pup (B) and an exposed PND7 rat pup (C). Here again we depict **Hoechst33342** in blue, and **caspase3** in green, and **NeuN** in red for (B), (C), (D), and (E). An increase in the number of caspase3 positive cells in the sevoflurane exposed PND7 rat pup is noted, indicated by the white arrows comparing representative images of an unexposed thalamus (B) with an exposed thalamus (C). Also seen here is a closer view of a region in the thalamus with a cluster of apoptotic cells (D), allowing for

better identification of the nuclei through the Hoechst33342 staining. With higher magnification using 63× oil objective (E), we can clearly see the nuclei of caspase3 positive cells, appearing irregularly shaped, forming small rounded structures made up of chromatin bodies indicating the fragmenting DNA of dying cells. These are seen next to other normal appearing nuclei that do not stain for caspase3. Notice also an abnormally shaped nucleus in a cell staining positive for NeuN and negative for caspase3; this indicates that the caspase3 stain is specific for apoptotic cells, only during certain phases of apoptosis. Also notice in this image a cell with an abnormal nucleus staining positive for caspase3 and negative for NeuN, indicating that non-neuronal cells can also undergo apoptosis and stain positive for caspase3.

Table 1

Experimental Groups

Group	Anesthesia Exposure Yes/No	Age at Exposure or Control	Age at ¹ HMRS Acquisition
1	Yes	PND7	PND8 and 9
2	No	PND7	PND8 and 9
3	Yes	PND15	PND16 and 17
4	No	PND15	PND16 and 17

Four groups were used for these experiments; groups 1 and 2 were tested during time of peak vulnerability to anesthesia-induced neurotoxicity, while groups 3 and 4 were tested shortly after this vulnerability period. For those in groups 1 and 3, designated as 'exposure' groups, each rat was exposed to sevoflurane anesthesia for 5 hours on either PND7 for group 1 and PND15 for group 3. Those in groups 2 and 4, designated as 'unexposed', were kept away from their litter for 5 hours without any anesthesia exposure. All rats (exposed or unexposed) were tested using ¹HMRS on the two subsequent days, each of these acquisitions requiring a brief anesthesia exposure. (¹HMRS = proton magnetic resonance spectroscopy, PND = post-natal day)

Table 2

Metabolite concentrations from thalamus in exposed and unexposed rats from PND8 to PND9

Metabolites	Unexposed (N=21)		P-value	Exposed Rats (N=19)		P-value	Group*Age Interaction P-value
	PND8	PND9		PND8	PND9		
Glu	4.56±0.57	4.78±0.46	0.244	4.70±0.53	4.49±0.54	0.221	0.092
Ins	3.05±0.67	2.81±0.78	0.250	3.34±0.55	3.05±0.55	0.550	0.881
NAA	2.08±0.24	2.41±0.21	0.0001**	2.15±0.20	2.26±0.26	0.136	0.031*
GPC+PCh	0.54±0.20	0.58±0.16	0.510	0.60±0.17	0.46±0.11	0.020*	0.024*
Cr+PCr	4.38±0.56	4.66±0.41	0.081	4.47±0.25	4.46±0.54	0.905	0.141

Metabolite concentrations for PND7 exposed and unexposed groups are shown for the five key metabolites investigated. This shows a significant increase in NAA concentration from PND8 to PND9 in the unexposed group, as well as a significant decrease in total choline (GPC+PCh) concentration in the exposed group, with a significant Group*Age interaction as determined by two-way ANOVA analysis. This represents the normal increase in the neuronal marker NAA during brain maturation for the unexposed animals. The exposed animal group is identified by an inhibited increase in NAA, along with a significant decrease in total choline. (*Cr+PCr* = total creatine, *Glu* = Glutamine, *GPC+PCh* = total choline, *Ins* = myoinositol, *NAA* = N-Acetyl Aspartate, *PND*=post-natal day).

Table 3

Metabolite concentrations from thalamus in exposed and unexposed rats from PND16 to PND17

Metabolites	Unexposed (N=16)		P-value	Exposed Rats (N=14)		P-value	Group*Age Interaction P-value
	PND16	PND17		PND16	PND17		
Glu	7.00±0.54	7.32±0.85	0.180	6.81±0.83	7.31±0.83	0.062	0.575
Ins	2.37±0.48	2.33±0.49	0.838	2.19±0.30	2.44±0.37	0.008*	0.139
NAA	4.58±0.48	4.97±0.64	0.008*	4.45±0.66	5.00±0.60	0.003*	0.436
GPC+PCh	0.70±0.07	0.75±0.13	0.134	0.74±0.15	0.82±0.13	0.104	0.674
Cr+PCr	5.65±0.52	5.80±0.69	0.339	5.75±0.73	6.17±0.74	0.036	0.280

Metabolite concentrations for PND15 exposed and unexposed groups are shown for the five key metabolites investigated. This shows a significant increase in NAA concentration from PND16 to PND17 in both the exposed and unexposed groups; this is in contrast to the findings that NAA increase was inhibited in the PND7 exposed group. Also the significant decrease in total choline (GPC+PCh) concentration in the exposed PND7 group was not found in this older animal group. There was no significant Group*Age interaction effect as determined by two-way ANOVA analysis for these groups. (*Cr+PCr* = total creatine, *Glu* = Glutamine, *GPC+PCh* = total choline, *Ins* = myoinositol, *NAA* = N-Acetyl Aspartate, *PND*=post-natal day).