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Full Length Article

Early life exposure to extended general anesthesia with isoflurane and nitrous oxide reduces responsivity on a cognitive test battery in the nonhuman primate

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ABSTRACT

Despite the widespread use of general anesthesia, a growing body of research suggests that anesthesia exposure early in life may be associated with acute neurotoxicity and lasting behavioral changes. To better evaluate the risk posed by early life anesthesia on cognitive development, infant rhesus monkeys were exposed to an anesthesia regimen previously shown to be neurotoxic and their cognitive development was subsequently measured using a translational operant test battery. On postnatal day 5 or 6, animals were exposed to 8 h of isoflurane (n = 6, 1% isoflurane in a vehicle gas of 70% nitrous oxide and 30% oxygen) or a control condition (n = 8). Starting at 7 months of age, the monkeys were continuously trained and assessed on the NCTR Operant Test Battery (OTB). The OTB consists of cognitive tests which also exist in near identical forms for use in rats and humans, and includes tests of learning, memory, color discrimination, and motivation. Monkeys previously exposed to anesthesia showed a clear decrease in responding in a measure of motivation, as well as a lower response rate in a learning task. These data further support the hypothesis that prolonged anesthesia early in life may increase the risk of developing cognitive impairments later in life.

1. Introduction

Despite its frequent use, a growing body of research suggests that general anesthesia early in life is associated with an increased incidence of developmental cognitive disorders. For example, children who had received multiple exposures to general anesthesia before the age of four (Wilder et al., 2009) were 60% more likely to be diagnosed with a learning disability. A related study described a significantly increased risk of a learning disability or ADHD diagnosis in children who had received multiple anesthesia exposures before the age of two (Flick et al., 2011; Sprung et al., 2012). More recently, these results were replicated in an independent cohort of children, with the results further suggesting the potential for general anesthesia duration-dependent effects (Hu et al., 2017). Unfortunately, it is difficult to separate the contribution of the need for surgery, or the effects of surgery, from the effects of anesthesia on neurotoxicity.

Perhaps the strongest *experimental* evidence for anesthesia-induced toxicity comes from studies in the rhesus monkey where it has been demonstrated that ketamine, isoflurane, sevoflurane, and propofol all have neurotoxic potential. For example, early work with ketamine demonstrated that a 24-hour exposure at gestational day (GD) 122 or post-natal day (PND) 5-but not PND 35-was capable of inducing neurotoxicity as measured by Fluoro-Jade C staining (Slikker et al., 2007). While 24 h is a long treatment duration, the dissociation between the effects seen at GD 122 and PND 5 and the lack of effect at PND 35 effectively highlight the importance of the "brain growth spurt" as a sensitive period for this phenomenon. Moreover, additional work with ketamine highlighted its potential to be demonstrably neurotoxic after a 9 h (Zou et al., 2009) or even 5 h exposure (Brambrink et al., 2012b). Further evidence for a period of sensitivity during the brain growth spurt was supported by the finding that ketamine on GD 120 induced twice as much degeneration as the same exposure on PND 6 (Brambrink et al., 2012b). Moreover, evidence of increased cell death at GD 120 was seen in numerous areas, but largely constrained to the caudate nucleus, globus pallidus, and thalamic regions by PND 7. More recently, it was established that isoflurane and propofol can induce neuronal toxicity at GD 120 and PND 6 (Brambrink et al., 2010; Creeley et al., 2013, 2014) after 5 h exposures (caspase-3 positive neurons), and sevoflurane has been shown to increase neuronal cell death (Fluoro-Jade C) after 9 h exposures (Liu et al., 2015). Similarly, isoflurane (1%) in

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combination with nitrous oxide (70%) has been shown to increase cell death after a 9 h exposure (Zou et al., 2011), while isoflurane or nitrous oxide on its own had no apparent effect (Zou et al., 2011). Further, these exposures appear to have functional consequences. For example, Coleman et al. (Coleman et al., 2017) demonstrated that monkeys exposed to multiple bouts of isoflurane-based anesthesia (5 h) showed delayed motor development at 1 month of life and evidence of an altered anxiety response one year later. Similarly, monkeys exposed to 24 h of ketamine have impairments on operant tasks of learning and memory for several years after the initial exposure (Paule et al., 2011). These data suggest that the neurotoxicity induced by anesthesia exposure early in life may have long-lasting consequences on cognitive function.

An inherent challenge in assessing the impact of neurotoxic insults in *pre-clinical* models of human cognitive function is that it can be difficult to estimate how the observed changes relate to *clinical* endpoints such as IQ, academic achievement, or the incidence of developmental disorders. One approach to increase this relevance is to test nonhuman subjects (i.e., rodents or NHPs) and human subjects under similar conditions. By using such an approach, it is possible to show how a translational measure, that is, one that should tap into the same neurobiology across species, is related to a clinically relevant endpoint, such as IQ, in humans. An example of an assessment tool that is useful across species is the National Center for Toxicological Research's Operant Test Battery (OTB). The OTB is a cognitive test battery that exists in nearly identical forms for children and NHPs (children received nickel reinforcers while NHPs received banana flavored food pellets) (Paule, 1990; Paule et al., 1988). Moreover, subtests of the OTB have been found to correlate with IQ, and are sensitive to the administration of stimulant medication in children with ADHD, highlighting the clinical relevance of this translational instrument (Baldwin et al., 2004; Chelonis et al., 2002; Paule et al., 1999). In NHPs, the OTB is sensitive to various acute pharmacological treatments such as MK-801 (Buffalo et al., 1994), LSD (Frederick et al., 1997), diazepam (Schulze et al., 1989), pentobarbital (Ferguson and Paule, 1993), and chlorpromazine (Ferguson and Paule, 1992). Acquisition of OTB performance can also be negatively impacted by chronic pharmacological treatments during training. For example, remacemide (Popke et al., 2002) and marijuana (Paule et al., 1992) alter acquisition behavior when given regularly during development Moreover, early life exposure to ketamine-induced general anesthesia (PND 5 or 6) caused impairments on several-sub tests of the OTB (Paule et al., 2011) when assessed even years later. Because of the high clinical relevance of the OTB and its demonstrated sensitivity to detecting toxicity during the developmental period, it is an ideal tool for modeling the potential neurotoxic effects of early life exposure to anesthesia.

For the OTB, animals are trained on several different tasks requiring a response to multiple manipulanda following the presentation of auditory and visual stimuli (Paule, 1990). Monkeys are initially trained on an Incremental Response Acquisition (IRA) task, designed to assess the ability to learn new information. The animal is required to learn a new sequence of lever presses (up to a length of six) daily to earn food pellet reinforcers. Shortly after training on the IRA begins, animals start training on a Progressive Ratio (PR) task to assess motivation and responsivity. For the PR task, animals are required to press a single designated lever to earn food pellets. However, with each subsequent trial, the number of presses required to earn a reinforcer increases by one. The number of total presses made and the rate of pressing can be used as measures of motivation. Once animals are reliably performing the PR task, they begin training on a conditioned position responding (CPR) task, in which subjects learn to make a response at a left press-plate or a right press-plate in response to one of four color cues. Finally, animals are trained on a delayed matching-to-sample (DMTS) task. For the DMTS task, subjects are shown a geometric symbol (e.g., circle, triangle) and respond to it. After experiencing a short delay, the animal is presented with the previously observed image, as well as two "new" images. The animal is required to select the previously observed image to earn a food pellet reinforcer. When animals are fully trained, they perform PR and IRA on one test day, and CPR and DMTS on alternate test days. The training durations necessary and the performance of individual tasks are used as measures of cognitive performance. In this way, a composite performance score as well as a series of sub-scores can be generated which are thought to be more relevant to specific aspects of cognitive function (i.e., memory or motivation).

While it has been previously demonstrated that exposure to isoflurane (1%) with nitrous oxide (70%) induces neurodegeneration, the clinical impact of these exposures and the resultant cell death has not been determined. The goal of this work is to determine if isoflurane with nitrous oxide will negatively impact performance on a translational cognitive test battery, including tasks shown to correlate with IQ in humans (CPR and IRA). To gain better insight into the functional consequences of developmental isoflurane and nitrous oxide exposures, rhesus monkeys were exposed (PND 5 or 6) to 8 h of isoflurane (1%) with nitrous oxide (70%) or a control condition. We elected to study isoflurane and nitrous oxide in combination for several reasons. (1) Several large-scale studies investigating the impact of pediatric anesthesia identified nitrous oxide as the most frequently used form of general anesthesia (Hu et al., 2017; Wilder et al., 2009). Under normal atmospheric conditions nitrous oxide alone is not sufficient to produce full anesthesia, however it is effective at increasing the absorption of an anesthetic via the "second gas effect", essentially lowing the minimum alveolar concentration of a gaseous anesthetic (Epstein et al., 1964). Accordingly, a "second" anesthetic agent is still required to achieve full anesthesia. In this instance isoflurane (1%) in combination with nitrous oxide (70%) was used to maintain anesthesia. While perhaps less commonly used then other inhaled anesthetic agents (Hu et al., 2017), isoflurane is included on the World Health Organization's Model list of (Essential Medicines (16th list) (2010)), highlighting its global relevance. At approximately 7 months of age, animals started training on the OTB. Animals were generally tested for 5 days a week for 50 min each test day. Task accuracy, completion rate, and various response latencies served as our primary measures for this work.

It has previously been shown that this anesthesia regimen (1% isoflurane with 70% nitrous oxide) will cause increased cell death in several brain regions (Zou et al., 2011), and that extended ketamine exposures caused broad deficits on the OTB (Paule et al., 2011). We anticipate that animals treated with isoflurane (1%) and nitrous oxide (70%) will show impairments on most portions of the OTB. A potential short-coming of this design is that by using isoflurane with nitrous oxide we will be unable to definitively attribute observed impairments to isoflurane, nitrous oxide, or synergistic effects of the two drugs. However, studying the drugs in combination is more reflective of patterns of clinical use, ultimately increasing the clinical relevance of these data.

2. Methods

2.1. Animals

16 rhesus macaques (Macaca mulatta, Indian origin) were used for this study. The control group consisted of 5 male and 3 female monkeys, while the isoflurane group consisted of 3 male and 5 female animals. Animals were exposed on PND 5 or 6. Animals were born in-house from our resident breeding colony and trained on site. All animal procedures were approved by the National Center for Toxicological Research's Institutional Animal Care and Use Committee and were conducted in full accordance with the PHS Policy on Humane Care and Use of Laboratory Animals (NCTR protocol number E07285.01).

2.2. Isoflurane exposure

Prior to the start of an exposure, all animals were administered

Table 1
Delayed used at each level of DMS training.

DMS Level	1	2	3	4	5	6
6	0.01	0.01	0.01	0.01	0.01	0.01
7	0.01	0.001	0.001	1	1	1
8	0.01	0.01	1	1	2	2
9	0.01	1	1	2	2	4
10	0.01	1	1	2	4	8
11	0.01	1	2	4	8	16
12	0.01	2	4	8	16	32
13	0.01	4	8	16	32	48
14	0.01	8	16	32	48	64
15	0.01	8	16	32	48	80

glycopyrrolate (0.01 mg/kg; I.M.; American Reagent, NY, USA) to reduce airway secretions. During the exposures, all animals were kept in an incubation chamber maintained at 37 °C. An isoflurane specific vaporizer (E-Z Anesthesia, PA, USA) was used to administer isoflurane (1.0%) in a vehicle gas combination of oxygen (30%) and nitrous oxide (70%) at a rate of 1 L/min delivered to the incubation chambers. The chambers were continually vented through activated charcoal filters. All animals were administered 5 ml of 5% dextrose solution every 2 h during the 8-hour exposure period via a gastric tube. The tube was removed after administration. Moreover, temperature, peripheral capillary oxygen (SpO2; N-395 Pulse Oximeter, Ca, USA), respiratory rate and expired CO_2 concentrations (Tidal Wave Hand-held Capnograph, Respironics, CA, USA) were measured throughout the exposure. Blood (ulnar vein, 0.25 ml) was collected every two hours for the measurement of plasma glucose (Ascensia Elite XL Blood Glucose Meter, Bayer Diagnostics, NY, USA) and the determination of venous blood gas concentrations (Rapid Lab, MA, USA). Animals were lightly restrained for the collection of blood samples in the control group. Control animals were kept in an incubation chamber for 8 h and exposed to warmed room air throughout. Vital parameters are reported in Table 1.

2.3. OTB testing

At approximately 7 months of age, all subjects were weaned and fitted with permanent collars to allow the use of catch poles for animal restraint and transportation. Prior to the beginning of testing, animals were acclimatized to the transport chairs for two weeks. During this period, animals were placed in the restraint/transport chairs for one hour daily (Mon-Fri) and offered banana flavored food pellets (190 mg dustless, Bio-Serv, New Jersey, USA). During this period, animals were weighed daily and their daily food allotment (standard nonhuman primate chow, Purina Mills, IM, USA) was adjusted weekly to ensure that animals gained approximately 0.1 kg/month. Animals were fed daily at approximately the same time and water was provided ad libitum in the home cage. This standard diet was supplemented with fresh fruit and multivitamins (USA Drug, AR, USA). Once OTB training began animals were tested for about 1 h a day, typically 5 days a week. Animals were between 3.5 and 4 years old at the end of this study.

The method used for OTB training was nearly identical to that used in previous studies (Paule, 1990; Paule et al., 2011). See Fig. 1 for an illustration of an OTB test panel. The order in which the tasks from the Operant Test Battery (OTB) were introduced was as follows: Incremental Repeated Acquisition (IRA); Conditioned Position Responding (CPR); Progressive Ratio (PR); and finally, Delayed Matching-to-Sample (DMTS). Once training on any given task was completed (i.e., the subject was performing the task using the final testing parameters), the subject continued to perform that task under the final task parameters. In total, about 3 years of behavioral testing data was included for each monkey. Monkeys were 3.5–4 years old at the end of this study.

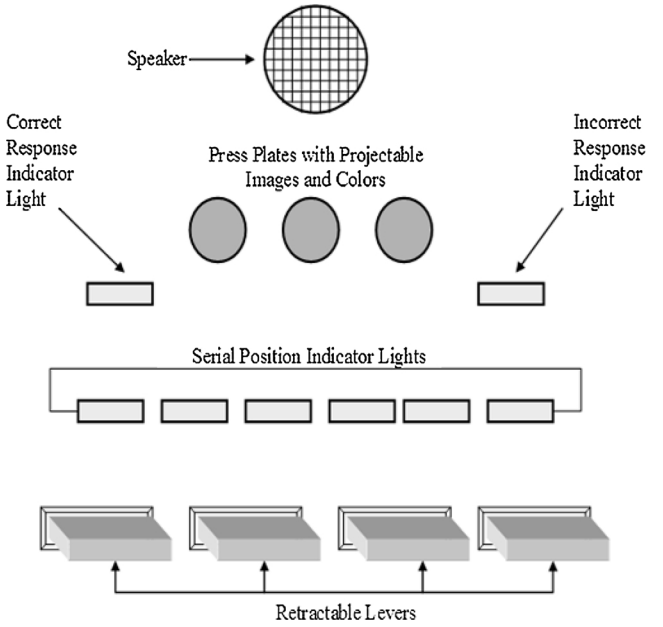

Fig. 1. A schematic of the OTB testing apparatus.

2.4. Training IRA

Training IRA level 1 (TIRA1). OTB training began with the Training IRA task. Initially, each IRA session lasted 50 min. A series of four levers were extended from the panel and a response on any of these levers resulted in reinforcer delivery. Once the subject earned 40 reinforcers (cumulative, across sessions), the subject progressed to the next IRA training level.

Training IRA level 2 (TIRA2). For this level, all four levers were presented, but a press on only three levers (selected randomly each test sessions) resulted in reinforcer delivery (one lever was inactive). Once the subject earned 40 reinforcers across sessions, the subject progressed to the next IRA training level.

Training IRA level 3 (TIRA3). This level was the same as TIRA2, except two of the four levers were now inactive, chosen randomly each test session. Once the subject earned 40 reinforcers across sessions, the subject progressed to the next IRA training level.

Training IRA level 4 (TIRA4). This level was the same as TIRA3, except three of the four levers were now inactive, chosen randomly each test session. Once the subject earned 40 reinforcers across sessions, the subject progressed to the full IRA task.

2.5. Full IRA task

The full IRA task began with the extension of the four response levers and the illumination of the rightmost of 6 horizontally aligned stimulus lights, which were used to indicate which level of the required response chain was in effect. The subject had to learn which of the four levers to press that would illuminate the correct response indicator light for 1 s and earn a reinforcer. If the subject made an incorrect response, the incorrect response indicator light was illuminated for 2 s and responses during that time had no programmed consequences. After the subject earned 20 pellets on this one-lever sequence, a 30 s delay occurred during which all of the stimulus lights were dark and responses had no programmed consequences. Afterwards, the stimulus light that was second from the right was illuminated indicating that the IRA level 2 was in effect. Here, another lever had to be pressed before pressing the original correct lever (i.e., now a 2-lever sequence). The subject had to determine which of the four response levers was correct for this level. As before, an incorrect response resulted in the illumination of the incorrect response indicator light for 2 s: if the correct lever was pressed,

the correct response indicator light was illuminated for 1 s, after which the 2nd to the right stimulus light was darkened and the rightmost stimulus light was illuminated to indicate the subject was now at IRA level 1. The subject had to then press the correct lever for this stimulus light in order to get a reinforcer and start the next trial where the stimulus light second to the right was once again illuminated. After earning 20 pellets on this 2 lever sequence, the subject was then presented with a three lever sequence, and so on until the subject completed 20 trials on a 6-lever sequence, or the session had ended after 50 min.

2.6. Training CPR

Animals started training on the CPR task after having completed 3 sessions of the full IRA task in which the subject earned 20 reinforcers (i.e., completed IRA level 1). At this point, the IRA task was presented every other test day and the CPR task was presented for 50 min on test days when the IRA task was not presented.

Training CPR level 1. To begin training on the CPR task, the three press-plates were illuminated the same color, selected from blue, green, yellow, red or white. A response on any of the press-plates resulted in the delivery of a reinforcer and the continued presentation of one of the four colors on all four press-plates. This continued until 100 reinforcers, cumulative across sessions, were earned.

Training CPR level 2. For this training level, the center press-plate and one of the side press-plates was illuminated. If the left press-plate was illuminated, then both the center and the left press-plate was illuminated red or yellow. If the right press-plate was illuminated, then both the center and right press-plate were illuminated green or blue. A response on one of the illuminated press-plates resulted in the delivery of a reinforcer and the start of the next trial. A response to a press-plate that was not illuminated had no programmed consequences. Subjects remained on this training level until 100 reinforcers, cumulative across sessions, were earned.

Training CPR level 3. On this training level, a trial began with the illumination of the center press-plate with one of four colors (red, yellow, blue, or green). Once the subject pressed the illuminated press-plate, one of the side press-plates was illuminated with the same color. The left press-plate was illuminated if the center press-plate had been red or yellow, and the right press-plate was illuminated if the center press-plate had been illuminated blue or green. A response to the illuminated side press-plate resulted in reinforcer delivery and the re-illumination of the center press-plate. Subjects remained on this training level until 100 reinforcers, cumulative across sessions, were earned.

Training CPR level 4. The procedure here was similar to that for training level 3 except that the side press-plate was illuminated white instead of the same color as was previously displayed on the center press-plate. After 100 reinforcers were earned, cumulative across sessions, the subject advanced to training level 5.

Training CPR level 5. The procedure for this training level was similar to that for level 4, except that after a response to the illuminated center press-plate, the center press-plate was darkened and both side press-plates were illuminated white. In order to receive a reinforcer and be presented a new color on the center press-plate, the subject had to press the left press-plate if the previous color displayed on the center press-plate was red or yellow, and press the right press-plate if the previous color displayed on the center press-plate was blue or green. If the subject pressed the incorrect side press-plate, there was a 10 s timeout and the same color was displayed on the center press-plate as was presented on the previous trial (error correction permitted). Subjects remained on this training level until a total of 1000 reinforcers were received after which the subject was presented with the full CPR task.

2.7. Full CPR task

The full CPR task was identical to that at CPR training level 5 except the same color was not repeated on the next trial if the subject responded on the incorrect side press-plate (error correction was not permitted). Furthermore, the task duration was reduced to 10 min and the subject could earn a maximum of 60 reinforcers during a session.

2.8. Full PR task

After 3 sessions of training CPR, subjects began performing the PR task on the same days as the IRA task. At this time, the IRA task was shortened from 50 min to 40 min and was performed 1 min after the PR session ended. Given that the subjects were already pressing levers for the IRA task, there was no formal lever-press training for the PR task. The PR task began with the extension of only the far right response lever. Reinforcers were delivered for responses on this lever under a PR 1 + 1 schedule. A press on this lever resulted in the delivery of a reinforcer, and each reinforcer required one additional press beyond what was required previously. In other words, the first reinforcer required 1 press, the second, 2 presses, the third 3 presses, etc. The task continued for 10 min or until 100 reinforcers were received.

2.9. DMTS training

Once the full task for CPR was begun, training on the DMTS task commenced. DMTS training began 1 min following the full CPR task and lasted for 40 min. At this point, subjects were performing the PR and IRA task on one test day and the CPR and DMTS task on alternate test days (all weekdays, M-F).

Training DMTS level 1. To begin, an identical image selected from seven possible geometric shapes was displayed on each of the three press-plates. A press on any of the press-plates resulted in reinforcer delivery and the same or a new image (selected randomly) being presented. A subject remained on this level until a total of 100 reinforcers were earned, cumulative across sessions.

Training DMTS level 2. For this training level, the center press-plate and one of the side press-plates were illuminated with the same symbol. A response on one of the illuminated press-plates resulted in the delivery of a reinforcer and the start of the next trial. A response to a press-plate not illuminated had no programmed consequences. Subjects remained on this training level until 100 reinforcers, cumulative across sessions, were earned.

Training DMTS level 3. A trial began with the illumination of the center press-plate with one of the seven symbols. After the subject pressed the center press-plate, it was darkened and then one of the three press-plates was illuminated with the same symbol. When the subject pressed the illuminated press-plate, a reinforcer was delivered and a new trial began. Responses on press-plates that were not illuminated had no programmed consequences. The subject remained on this training level until a total of 100 reinforcers were earned across sessions.

Training DMTS level 4. Training for this level was the same as for level 3, except two press-plates were illuminated following a successful response to the stimulus displayed on the center press-plate. For this level, one of the stimuli was the same (matched) as the ‘sample’ stimulus that was presented on the center press-plate at the start of the trial and the other was a different (non-matching) stimulus. If a subject pressed the press-plate that contained the matching shape, the subject received a reinforcer and the next trial began with another shape being presented on the center press-plate. A response to the non-matching shape resulted in a 10 s timeout followed by the same shape being displayed on the center-press plate as was presented on the previous trial (i.e., the matching problem was repeated). The subject remained on this training level until a total of 100 reinforcers were earned across sessions.

Table 2
A summary of vital parameters during treatment. A main effect of Isoflurane was detected on body temperature ($p < 0.001$). Significant interactions were observed between "time" and "treatment" on respiration rate, body temperature, and blood glucose. Sample size varies owing to missing measurements and the use of a repeated measures ANOVA.

		Respiration Rate (breaths per min)						Heart Rate (beats per min)					
Group		0 h	2 h	4 h	6 h	8 h		0 h	2 h	4 h	6 h	8 h	
Control n = 8	Avg.	84.9	90.5	78.0	97.1	73.8	n = 8	104.5	217.9	208.6	201.1	195.0	
	S.E.M.	7.0	9.1	12.6	10.7	7.6		0.9	18.2	15.7	13.1	16.3	
Isoflurane n = 7	Avg.	88.0	98.1	102.9	83.6	69.1	n = 8	112.5	173.4	181.1	172.0	169.8	
	S.E.M.	7.5	9.8	13.4	11.4	8.1		0.9	18.2	15.7	13.1	16.3	
		Sp02 (%)						Body Temperature (C°)					
Group		0 h	2 h	4 h	6 h	8 h		0 h	2 h	4 h	6 h	8 h	
Control n = 8	Avg.	97.6	96.3	97.9	96.7	96.4	n = 8	36.2	38.1	37.9	37.7	38.1	
	S.E.M.	0.7	1.1	0.9	1.4	0.9		0.5	0.2	0.3	0.3	0.4	
Isoflurane n = 8	Avg.	97.3	97.6	98.0	95.0	97.2	n = 8	36.2	34.2	34.0	33.9	34.5	
	S.E.M.	0.7	1.1	0.9	1.4	0.9		0.5	0.2	0.3	0.3	0.4	
		Systolic BP (mmHg)						Diastolic BP (mmHg)					
Group		0 h	2 h	4 h	6 h	8 h		0 h	2 h	4 h	6 h	8 h	
Control n = 8	Avg.	107.6	111.3	105.4	121.3	96.1	n = 8	84.9	90.5	78.0	97.1	73.8	
	S.E.M.	8.0	9.5	13.3	12.1	8.7		7.0	9.1	12.6	10.7	7.6	
Isoflurane n = 7	Avg.	105.1	113.0	124.4	106.4	101.1	n = 7	88.0	98.1	102.9	83.6	69.1	
	S.E.M.	8.6	10.1	14.2	12.9	9.4		7.5	9.8	13.4	11.4	8.1	
		Blood Glucose (mg/dl)						Blood pH					
Group		0 h	2 h	4 h	6 h	8 h		0 h	2 h	4 h	6 h	8 h	
Control n = 7	Avg.	68.4	66.6	56.6	47.6	45.7	n = 7	7.2	7.2	7.3	7.2	7.3	
	S.E.M.	6.4	7.9	6.7	9.0	5.8		0.04	0.03	0.03	0.03	0.02	
Isoflurane n = 8	Avg.	70.4	65.4	60.6	75.9	62.9	n = 6	7.2	7.3	7.2	7.3	7.3	
	S.E.M.	6.0	7.4	6.3	8.4	5.4		0.04	0.03	0.04	0.04	0.02	
		Expired CO2 (mmHg)											
Group		0 h	2 h	4 h	6 h	8 h							
Control n = 7	Avg.	14.2	14.2	13.6	14.0	12.0	n = 5						
	S.E.M.	2.5	2.5	2.0	1.5	2.3							
Isoflurane n = 8	Avg.	16.0	13.7	14.0	15.3	13.7	n = 3						
	S.E.M.	3.2	2.6	2.0	2.9	5.4							

Training DMTS level 5. The procedure for this level was the same as for level 4, except that after an effective response on the center press-plate with the sample stimulus, all three press-plates were illuminated with a different symbol, only one of which matched the sample that has been displayed on the center press-plate. The subject remained on this level until 1000 reinforcers cumulative, across sessions, were earned.

DMTS delay training. The procedure for this level of training was the same as for level 5, except if an incorrect response was made when the three choice stimuli were presented, the same matching problem was not repeated on the next trial. Time delays between the response on the center press-plate when the matching stimulus was displayed and the presentation of the three choice stimuli were introduced at this point. Up to six different delays were possible with the initial presentations, all 0.01 s. These delays were changed when the subject attained 3 consecutive sessions in which 50 or more reinforcers were earned (See Table 2 for delay levels). The final delays used were 0.01, 8.0, 16.0, 32.0, 48.0, and 64.0 s. After this set of delays, the subject was then switched to the final parameters for the DMTS task.

2.10. Full DMTS task

The full task for DMTS was the same as that for the DMTS delay training with the final values for the delays of having an equal probability of occurrence. The final delay values were 0.01, 16.0, 32.0, 48.0, 64.0, and 80.0 s.

Analyses of the OTB performance measures and body weights were conducted using SAS, version 9.3. For these analyses, a three-way ANOVA was utilized to examine the main effects of treatment (prior isoflurane exposure or control), sex, and blocks of sessions and their interactions. Blocks of sessions were created by averaging data across 5 session periods (typically 2 weeks) for each animal for each task. In instances where a significant effect of treatment, or a treatment by block interaction, was observed, independent sample t-tests were also conducted to compare the performance of isoflurane exposed monkeys and control monkeys for each dependent variable at each session block. To reduce the likelihood that the accuracy data for the CPR or IRA tasks would be skewed due to a low number of responses, at least 5 reinforcers had to be earned or a minimum of 10 trials completed in order for accuracy data to be included in the analysis for any given session. Latency data (msec) was Log10 transformed prior to analysis. The variables analyzed were overall OTB training score and DMTS training score, IRA accuracy, IRA percent task completed, IRA effective response rate (total responses / total time when it was possible to make a correct response in s), CPR accuracy, CPR percent task completed, CPR observing latency (msec Log10), CPR choice latency (msec Log10), CPR effective response rate (total responses / total time when a response could be recorded), PR total reinforcers earned, PR total presses, and PR effective response rate (total responses / total time when it was possible to make a response which would count towards earning a reward in z). For the sake of brevity, only statistically significant effects are reported in the results section. A complete statistical analysis can be found in Tables 2–7. All Significant differences on specific treatment blocks are graphically illustrated on the relevant figure. An OTB training score was developed to monitor progress on training for each subject and served as a metric to compare this progress across groups. This method of quantifying training progression was described in previous research (Paule et al., 2011). Monthly body weights were also recorded and analyzed. Measurements of vital parameters taken during exposure were analyzed using a two-way repeated measure ANOVA, using "treatment" as an independent factor and "time of measurement" as a repeated measure (Statistica 64, version 13). Vital parameters were recorded at the onset of exposure and at two-hour intervals during the exposure (0 h, 2 h, 4 h, 6 h, and 8 h). These included percent peripheral oxygen saturation (SpO2), expired carbon dioxide (mmHG, Expired

Table 3
Detailed results from statistical analysis of vital parameters during exposure.

SpO2			
	DF	F	P
treatment	1, 14	0	0.97
time	4, 56	1.85	0.13
treatment*time	4, 56	1.01	0.4
Resp. Rate			
	DF	F	P
treatment	1, 14	0.65	0.43
time	4, 56	0.62	0.64
treatment*time	4, 56	5.4	< 0.001
Systolic blood pressure			
	DF	F	P
treatment	1, 13	0.033	0.85
time	4, 52	0.96	0.43
treatment*time	4, 52	0.79	0.53
blood glucose			
	DF	F	P
treatment	1, 13	1.77	0.2
time	4, 52	2.7	0.04
treatment*time	4, 52	2.92	0.029
temperature			
	DF	F	P
treatment	1, 14	102.8	< 0.001
time	1, 14	0.7	0.6
treatment*time	4, 56	14	< 0.001

Expired CO2			
	DF	F	P
treatment	1, 6	0.08	0.77
time	4, 24	0.84	0.5
treatment*time	4, 24	0.27	0.89
Heart Rate			
	DF	F	P
treatment	1, 14	2.02	0.17
time	4, 56	33.6	< 0.001
treatment*time	4, 56	2.3	0.069
Diastolic blood pressure			
	DF	F	P
treatment	1, 13	0.15	0.69
time	4, 52	2.1	0.09
treatment*time	4, 52	1.37	0.25
pH			
	DF	F	P
treatment	1, 11	1.7	0.22
time	4, 44	1.6	0.18
treatment*time	4, 44	0.5	0.76

CO2), respiratory rate (breaths per minute), heart rate (beats per minute), systolic and diastolic blood pressure (mmHG), blood glucose (mg/dl), blood pH, and temperature (C°).

3. Results

No significant effects of “treatment” or “time” by “treatment” were observed for SpO2, expired CO2, systolic and diastolic blood pressure, or blood pH. However a significant interaction between treatment and time was observed on respiratory rate ($F(4, 56) = 5.4$, $P < 0.001$). A significant interaction between treatment and time was observed on blood glucose levels ($F(4, 52) = 2.92$, $P = 0.029$). A main effect of treatment ($F(1, 14) = 102$, $P < 0.001$) and a treatment by time interaction ($F(4, 56) = 14$, $P < 0.001$) was observed on body temperature. See Tables 2 and 3 for additional details.

3.1. OTB training score

No significant effects were seen on the overall OTB training score (Fig. 2A, Table 4).

3.2. DMS training score

No significant effects were seen (see Fig. 2B, Table 4).

3.3. IRA

Analysis of IRA response rate indicate a significant interaction of isoflurane treatment with session block (lower responding as a result of isoflurane treatment, ($F(55, 605) = 2.42$, $P < 0.001$; Fig. 3C). Moreover a significant effect of isoflurane treatment and sex was detected on accuracy ($F(1, 11) = 5.0$, $P = 0.047$, Fig. 3A). An additional two way ANOVA including only the female animals indicated a near significant effect of isoflurane treatment ($F(1, 6) = 4.24$, $P = 0.085$), and a significant interaction between isoflurane treatment and block ($F(55, 330) = 1.55$, $P = 0.011$, Fig. 3D). See Table 5 for detailed statistical results.

3.4. CPR

Analysis of CPR choice latency indicated a significant interaction of

Table 4
Detailed results from statistical analysis of the OTB training score, DMS training score, and body weights.

OTB training score			
	DF	F	P
treatment	1, 10	1.68	0.22
sex	1, 10	0.08	0.78
block	35, 350	119.8	< 0.001
treatment*sex	1, 10	0.18	0.67
block*treatment	35, 350	0.84	0.72
block*sex	35, 350	0.55	0.98
block*treatment*sex	35, 350	0.48	0.99
Body wieghts			
	DF	F	P
treatment	1, 14	3.33	0.093
sex	1, 10	0.01	0.91
month	36, 432	331.68	< 0.001
treatment*sex	1, 12	0.96	0.34
month*treatment	36, 432	0.67	0.93
month*sex	36, 432	0.64	0.94
block*treatment*sex	36, 432	1.17	0.23

DMS training score			
	DF	F	P
treatment	1, 12	0.37	0.55
sex	1, 12	0.1	0.75
block	16, 192	95.57	< 0.001
treatment*sex	1, 12	1.68	0.21
block*treatment	16, 192	0.33	0.99
block*sex	16, 192	0.79	0.69
block*treatment*sex	16, 192	1.5	0.1

Table 5
Detailed results from statistical analysis of the IRA training.

Accuracy

	DF	F	P
treatment	1, 11	2.14	0.17
sex	1, 11	10.71	0.007
block	55, 605	12.72	< 0.001
treatment*sex	1, 11	5	0.047
block*treatment	55, 605	1.35	0.051
block*sex	55, 605	2.65	< 0.001
block*treatment*sex	55, 605	1.25	0.11

Effective response rate

	DF	F	P
treatment	1, 11	3.54	0.08
sex	1, 11	7.03	0.02
block	55, 605	3.18	< 0.001
treatment*sex	1, 11	2.54	0.13
block*treatment	55, 605	2.42	< 0.001
block*sex	55, 605	2.7	< 0.001
block*treatment*sex	55, 605	2.14	< 0.001

Percent task completed

	DF	F	P
treatment	1, 11	1.46	0.25
sex	1, 11	9.5	0.01
block	55, 605	7.86	< 0.001
treatment*sex	1, 11	2.98	0.011
block*treatment	55, 605	1.15	0.21
block*sex	55, 605	4.01	< 0.001
block*treatment*sex	55, 605	1.15	0.21

isoflurane treatment and session block (F(43, 516) = 1.47, P = 0.029). However, no significant differences between two groups were observed at any specific time point (Fig. 4B). See Table 6 for detailed statistical results.

3.5. PR

There was a main effect of isoflurane treatment in the analysis of total number of PR responses and indicated the isoflurane exposed group exhibited reduced responding (F(1, 12) = 8.29, P = 0.0138; Fig. 5A). This reduction in responding interacted with session block (F(55, 660) = 1.46, P = 0.019). The reduced rate of lever pressing in the isoflurane treated group was reflected in number of reinforcers earned in which the isoflurane treated group earned significantly fewer (F(1, 12) = 7.06, P = 0.021). See Table 6 for detailed statistical results.

Body Weight

There were no significant effects of isoflurane on body weight (see Fig. 6, Table 4).

4. Discussion

Several large clinical studies have established a link between the use of general anesthesia in pediatric populations and increased risks for various cognitive issues later in life. Most striking is the nearly 3-fold increase in the incidence of ADHD in children receiving general anesthesia at least twice in the first three years of life (Hu et al., 2017). While studies of this type are provocative, they cannot definitively determine if the increased incidence of those negative developmental outcomes resulted from the anesthesia, surgery, or the underlying need for surgery. To better evaluate the potential risk posed by general anesthesia, rhesus monkeys were exposed for 8 h to isoflurane plus nitrous oxide on PND 5 or 6 and then trained on a cognitive test battery starting at 7 months of age. Our primary finding is that isoflurane exposure decreased responding in the PR task designed to measure motivation

Table 6
Detailed results from statistical analysis of the CPR training.

Accuracy

	DF	F	P
treatment	1	0.01	0.93
sex	1	0.1	0.75
block	42	8.22	< 0.001
treatment*sex	1	2.03	0.17
block*treatment	42	1.03	0.42
block*sex	42	0.44	0.99
block*treatment*sex	42	1.18	0.2

Choice latency

	DF	F	P
treatment	1, 12	0.06	0.81
sex	1, 12	0.13	0.72
block	43, 516	11.04	< 0.001
treatment*sex	1, 12	0.68	0.42
block*treatment	43, 516	1.47	0.029
block*sex	43, 516	0.87	0.71
block*treatment*sex	43, 516	0.65	0.96

Effective response rate

	DF	F	P
treatment	1	1.37	0.26
sex	1	5.21	0.041
block	52	2.29	< 0.001
treatment*sex	1	0.02	0.9029
block*treatment	52	1.18	0.18
block*sex	52	1.92	< 0.001
block*treatment*sex	624	1.04	0.4

Percent task completed

	DF	F	P
treatment	1, 12	0.31	0.58
sex	1, 12	0.19	0.67
block	52, 624	3.12	< 0.001
treatment*sex	1, 12	0.56	0.46
block*treatment	52, 624	1.16	0.21
block*sex	52, 624	0.85	0.75
block*treatment*sex	52, 624	0.85	0.74

Observation latency

	DF	F	P
treatment	1, 12	4.23	0.062
sex	1, 12	0.29	0.6
block	43, 516	0.49	0.99
treatment*sex	1, 12	0.1	0.76
block*treatment	43, 516	0.96	0.54
block*sex	43, 516	1.59	0.011
block*treatment*sex	43, 516	0.89	0.66

Table 7
Detailed results from statistical analysis of PR training.

Total presses				Reinforcers earned			
	DF	F	P		DF	F	P
treatment	1, 12	8.29	0.013	treatment	1, 12	7.06	0.02
sex	1, 12	4.03	0.067	sex	1, 12	2.84	0.11
block	55, 660	6.81	< 0.001	block	55, 660	6.51	< 0.001
treatment*sex	1, 12	0.17	0.69	treatment*sex	1, 12	0.1	0.76
block*treatment	55, 660	1.46	0.019	block*treatment	55, 660	1.33	0.058
block*sex	55, 660	3.22	< 0.001	block*sex	55, 660	3.16	< 0.001
block*treatment*sex	55, 660	0.77	0.77	block*treatment*sex	55, 660	0.76	0.9

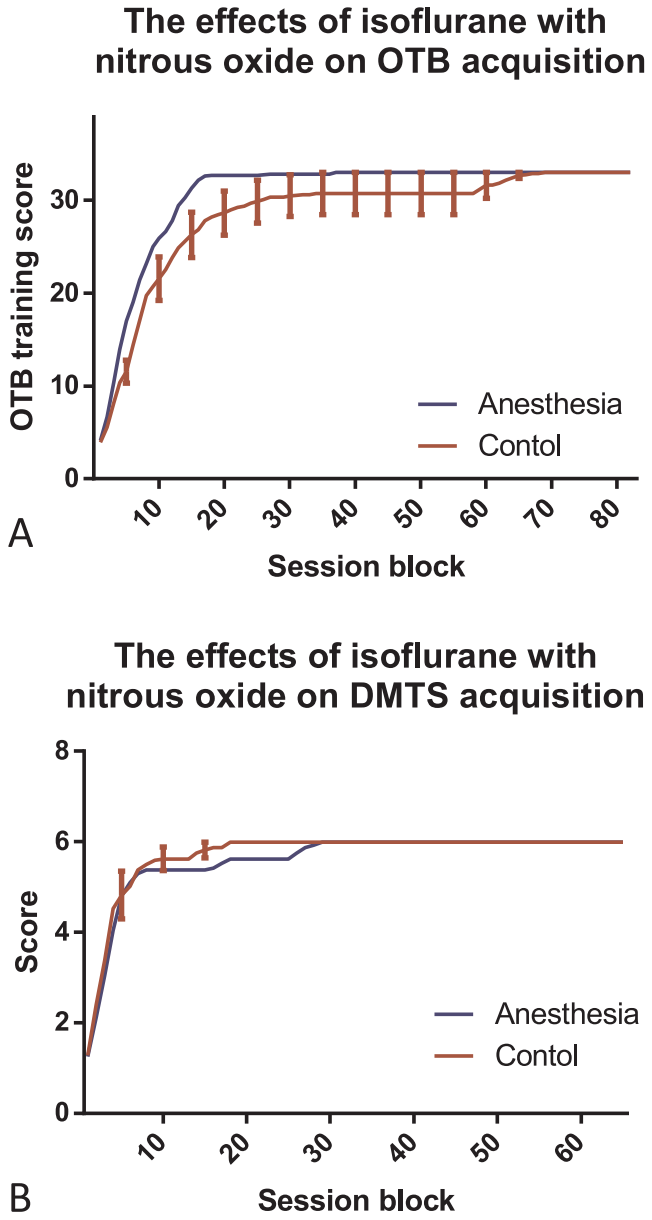

Fig. 2. No significant effects of treatment were seen on the OTB or DMS training scores (Fig. 2A,B). Lines represent 5 session block means while error bars represent 1 s.e.m (control only). Only every 5th s.e.m. is included to improve legibility.

and reduced responding in the IRA task. These data suggest that early life isoflurane exposure caused motivational impairment, and/or abnormalities in motor function. Additionally, female monkeys treated with isoflurane in combination with nitrous oxide were worse on IRA accuracy (interaction with block). This could be taken as evidence for an increased sensitivity to neurotoxicity in female animals (Boscolo et al., 2013; Jevtovic-Todorovic et al., 2001), but no other anesthesia treatment by sex interactions were observed in this data set. This data set does not support the notion that females may be more sensitive to the neurotoxic effects of anesthesia, nor was it powered to detect such differences.

We previously established that general anesthesia exposure (sevoflurane, isoflurane, and ketamine) within the first postnatal week caused an elevation of markers of neuronal cell death, such as increased levels of Fluoro-Jade C and Caspase 3 staining in the infant rhesus monkey (Liu et al., 2015; Slikker et al., 2007; Zou et al., 2011). These results are in agreement with other research indicating that isoflurane (Brambrink et al., 2012a, 2010; Creeley et al., 2014), ketamine (Brambrink et al., 2012b), and propofol (Creeley et al., 2013) may increase the incidence of neuronal degeneration in the perinatal rhesus monkey. However, increased incidence of markers of neuronal cell death does not mean that these exposures are causing clinically relevant changes in behavior. Early in life, the brain undergoes a period of rapid development, brain cells are rapidly maturing, and actively being pruned. This rapid rate of growth and pruning might provide a form of protection against the abnormal degeneration induced by anesthesia exposure. Because of this, it is necessary to determine if these exposures early in life can causing long-term functional changes.

In a previous study, we demonstrated that extended ketamine anesthesia during the first postnatal week induced long-lasting performance deficits on most measures of OTB performance (Paule et al., 2011), including IRA and CPR which have been shown to significantly correlate with IQ in children (Paule et al., 1999). While the exposure regimen used in that initial study was extreme, it was effective at highlighting the risk associated with perinatal anesthesia exposure and indicated the sensitivity of the OTB to anesthesia-related toxicity in the rhesus monkey. Unlike that previous research which demonstrated that ketamine disrupted multiple aspects of cognition, the effects with isoflurane reported here were largely limited to PR task performance in all animals. Animals treated with isoflurane showed a clear reduction in responding for several years on the PR task. PR tasks are classically used to study motivation and changes in the incentive value of reinforcers (Hodos, 1961; Markou et al., 2013), suggesting that these isoflurane-exposed monkeys may have reduced motivation or altered sensitivity to reinforcement.

Alternatively, the reduced rate of PR responding could be interpreted as a deficit in fine motor skills. Animals treated with Isoflurane were also found to make fewer responses on the IRA and CPR task, while being slower to respond during the “choice” phase of the CPR task. While this could be taken as further evidence of reduced motivation, performing the OTB does require a degree of manual dexterity. While no attempts have been made to validate the OTB as a measure of manual dexterity, it is possible that the observed deficits have their root in motor dysfunction rather than motivation. At this point in time it is impossible to determine if the observed results are motoric in nature, or instead represent a motivational deficit. However, support for anesthesia related motoric dysfunction can be found in the clinical

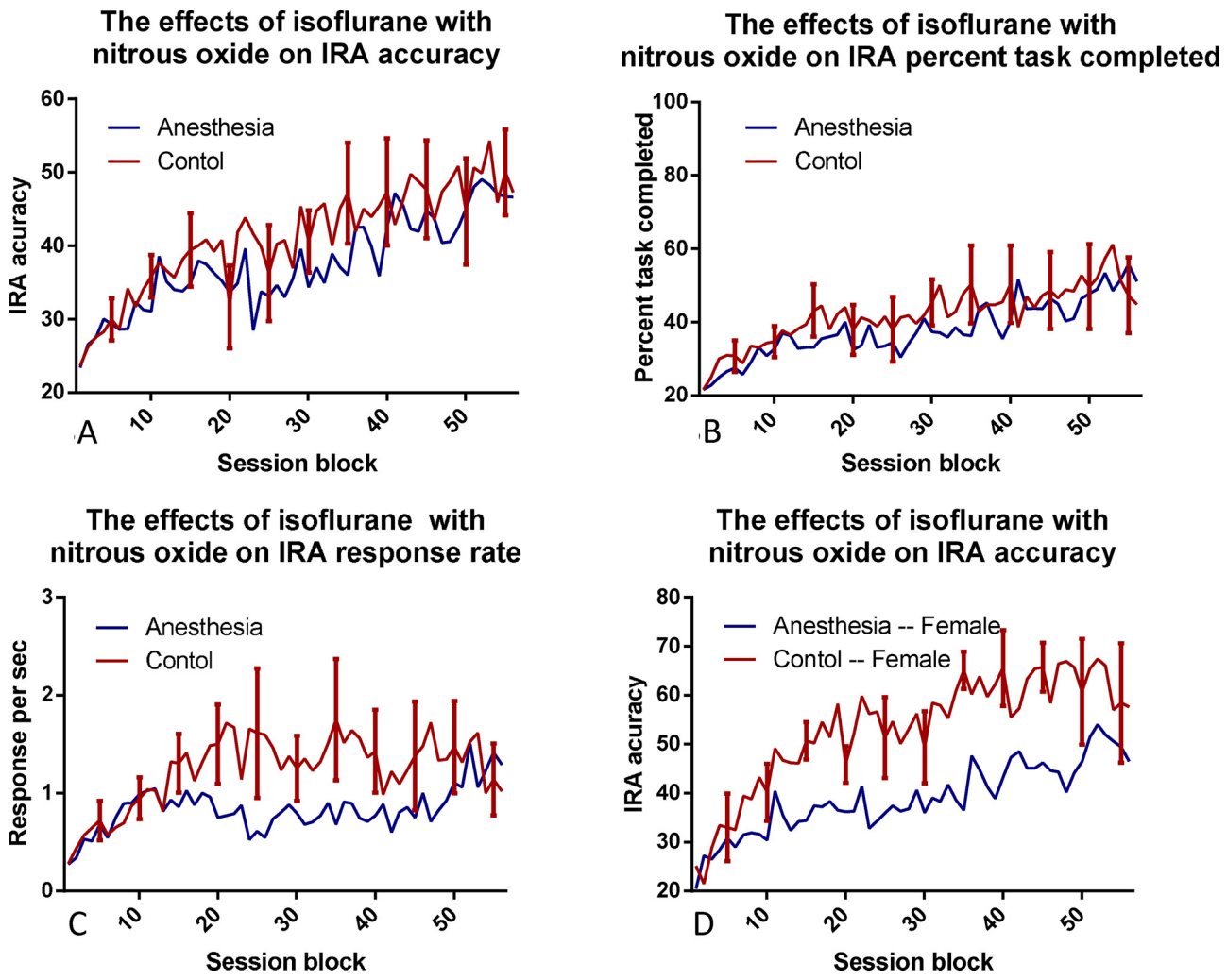

Fig. 3. No significant effects of Isoflurane were observed on IRA Accuracy, percent task completed, or response rate (Fig. 3A–C). A significant interaction between "sex" and "treatment" was observed (Fig. 3B). Further analysis indicated a significant interaction between "block" and "treatment" in female animals (Fig. 4D). Lines represent 5 session block means while error bars represent 1 s.e.m (control only). Only every 5th s.e.m. is included to improve legibility.

literature. In the Mayo Anesthesia Safety in Kinds (MASK) study, children who had been exposed to anesthesia before the age of three underwent intensive neuropsychological evaluations. One of the few significant effects of anesthesia exposure in the administered portion of the study were on Fine Motor Composite scores, providing clinical correlative evidence that general anesthesia before the age of three can compromise fine motor control (Warner et al., 2018). While PR performance was not associated with IQ in humans (Paule et al., 1999), treatment with isoflurane did cause changes in behavior that lasted for about a year and a half (Fig. 5).

We previously demonstrated that 24 h hours of ketamine anesthesia within the first week in the NHP increased markers of neuronal death and caused long-lasting cognitive impairments that have been associated with IQ in a clinical population (Paule et al., 2011). Like ketamine, extended exposure to isoflurane-based anesthesia will increase markers of neuronal death and will cause long lasting changes in behavior (Zou et al., 2011). Unlike ketamine (24 h), in this study isoflurane with nitrous oxide did not cause behavioral changes on endpoints that had previously been associated with IQ in a clinical population amongst all animals (although a significant interaction was observed between isoflurane treatment and block in just the female animals). Isoflurane plus nitrous oxide (8 h) appeared to be qualitatively less disruptive than extended ketamine exposure (24 h) on OTB performance, however the current findings are nonetheless concerning. Academic achievement is a cumulative process; competencies gained in the first year are necessary for success in the second year, and so on. As such, even transitory motivational (or motoric impairments) deficits in school-age children might have long-lasting implications for normal behavioral development and academic achievement. Moreover, while the 8 h exposure used here is long, it is not clinically irrelevant. Quality data on the length of anesthesia in children is limited, but in the MASK study 36% of the exposures in the "multiple exposure" cohort were 4 h or longer (Warner et al., 2018). Furthermore, despite different chemical structures and mechanisms of action (Anis et al., 1983; Harrison et al., 1993), both ketamine and isoflurane anesthesia induced neurodegeneration and caused performance deficits in the OTB (Liu et al., 2015; Paule et al., 2011; Slikker et al., 2007). These findings suggest that the process of general anesthesia, regardless of the drug used to maintain it, may be associated with adverse outcomes. Additional ongoing research with other general anesthetics will help to better characterize the relationship between perinatal anesthesia exposure, neuronal degeneration, and behavioral impairments.

Disclaimer

This document has been reviewed in accordance with United States Food and Drug Administration (FDA) policy and approved for publication. Approval does not signify that the contents necessarily reflect

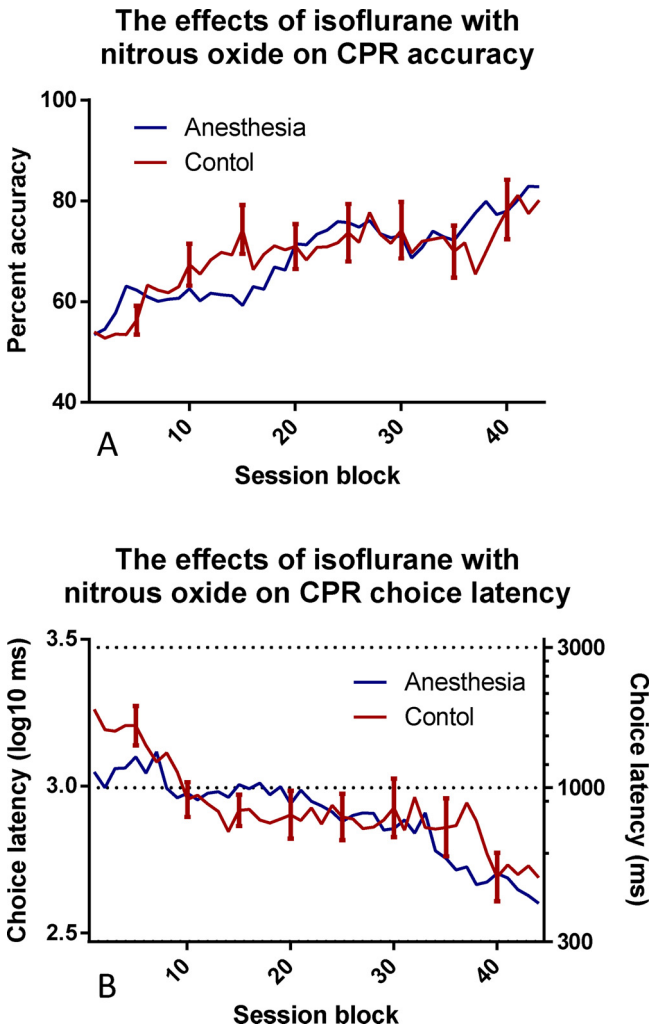

Fig. 4. Significant interactions between block and treatment were observed for CPR accuracy (Fig. 4A) and Choice latency (Fig. 4B). However post-hoc analysis did not indicate significant differences on any individual blocks. Lines represent 5 session block means while error bars represent 1 s.e.m (control only). Only every 5th s.e.m. is included to improve legibility.

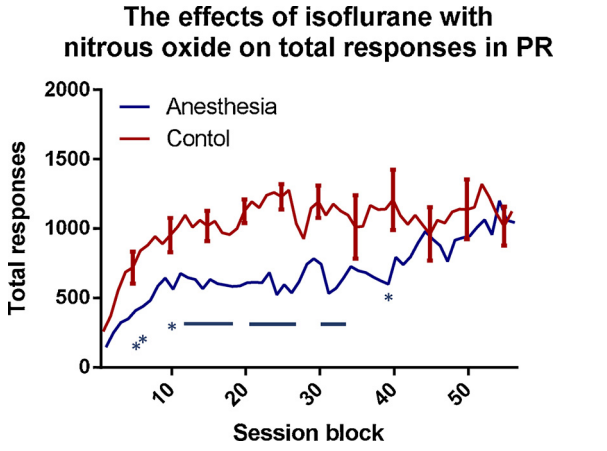

Fig. 5. A significant effect of Isoflurane treatment was observed with post-hoc analysis indicated significant differences ($p < 0.05$) in total presses made during a test session on sessions 5–6, 10, 12–18. 21–27, 30–33, and 39. Lines represent 5 session block means while error bars represent 1 s.e.m (vehicle only). Only every 5th s.e.m. is included to improve legibility. Significant data points are indicated with lines and "*".

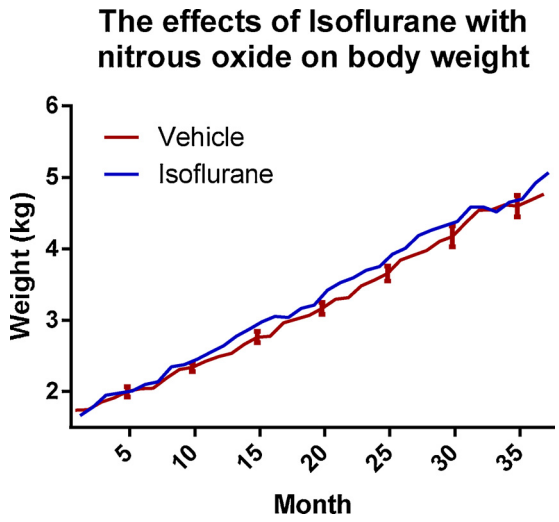

Fig. 6. No effects of isoflurane exposure were observed on weight gain during OTB training. Each data point represents a monthly average weight. The first data point corresponds to the beginning of OTB testing when animals were approximately 7 months old. Error bars represent one s.e.m (vehicle only).

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Conflict of interest

Authors John Chelonis, Mi Li, Joseph Hanig, and Merle Paule (retired) have worked for the Food and Drug Administration for the last 3 years and declare no conflict of interest. John Talpos has worked for the Food and Drug Administration for the last 32 months. Prior to that John Talpos worked for Janssen Pharmacutica (Belgium) and has no conflict of interest with the publication of this material.

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