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Acute Effects of Hypothermia and Inhalant Anesthesia on Ultrasonic Vocalizations and Neuroendocrine Markers in Neonatal Rats

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Neonatal rodents undergo anesthesia for numerous procedures and for euthanasia by anesthetic overdose. However, data regarding whether neonatal anesthesia is humane are limited. Hypothermia (cryoanesthesia) is the most commonly used anesthetic protocol for neonatal rats 10 d of age or younger. However, hypothermia has recently been restricted in several countries due to perceived painful effects, including pain on rewarming. Minimizing the potential pain and distress of neonates in research is imperative, although very challenging. Traditional validated and nonvalidated behavioral and physiologic outcome measures used for adult rats undergoing anesthesia are unsuitable for evaluating neonates. Therefore, we investigated the effects of several anesthetic methods on neonatal rats by using the innovative objective approaches of noninvasive ultrasonic vocalizations and more invasive neuroendocrine responses (i.e., serum corticosterone, norepinephrine, glucose). Our results show that hypothermia leads to heightened acute distress in neonatal rats as indicated by prolonged recovery times, increased duration of vocalizations, and elevated corticosterone levels, as compared with neonates undergoing inhalational anesthesia. We demonstrate that inhalational anesthesia is preferable to cryoanesthesia for neonatal rats, and researchers using hypothermia anesthesia should consider using inhalational anesthesia as an alternative method.

Abbreviations and Acronyms: PND, postnatal day; USV, ultrasonic vocalization

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Introduction

Neonatal rats, defined here as 14 d of age or younger, frequently undergo general anesthesia before translationally relevant survival procedures or the induction of preclinical models via experimental manipulations, including intracranial injections,[9] cardiac injury,[22] skin incisions,[2] nerve injuries,[35] thymectomies,[6] and gonadectomies,[1] or for euthanasia via anesthetic overdose. Ensuring the minimization of pain and distress in rodents used for research is a touchstone of veterinary clinical medicine, is codified in the AVMA's Veterinarian's Oath, and is integral to reproducible and reliable research studies. Although numerous well-evaluated anesthesia or euthanasia options are available for postweaning and adult rats,[12,29,32,34] resulting in revisions to the *AVMA Guidelines on Euthanasia* in 2020,[21] no standard protocols are available for anesthetic use in neonatal rodents. Until recently,[16] various anesthetic protocols for neonates had not been evaluated for safety and efficacy since the late 1900s.[8] Notably, preweaned animals have significantly different physiologic responses to commonly used anesthetic methods than do older animals, and neonates are more prone to anesthetic complications, including hypothermia and hypoglycemia.[16]

Hypothermia anesthesia, sometimes referred to as cryoanesthesia, is the most common form of anesthesia used in neonatal rats that are ≤ 10 d old.[8] This method is easily available and is often selected because these neonates are altricial and poikilothermic and can rapidly cool through surface cooling due to a high surface area:body mass ratio.[18] Young neonatal rodents are also relatively resistant to brain damage associated with cephalic circulatory arrest, thereby making them tolerant to extended periods of a 1 °C body temperature without known negative effects.[14] Offsetting the positive features of hypothermia are multiple potential risks, including ventricular fibrillation,[7] tissue hypoxia, and metabolic acidosis on warming,[38] all of which can negatively affect animal welfare and complicate the interpretation of research findings. Despite these adverse effects, hypothermia remains a standard means of anesthesia for neonatal rodents due to ease and speed of anesthesia induction, historic success, limited numbers of studies evaluating other anesthetic protocols, and lack of studies evaluating its acute effects on animal welfare. In accordance with the 3Rs principles (that is, replacement, reduction, and refinement), the use of hypothermia for anesthesia is now restricted in some parts of the world due to its perceived painful effects and the availability of other anesthetic modalities.[14]

Other methods of anesthesia commonly used in neonatal rats include gas anesthesia with sevoflurane or isoflurane,[16,26] administered either by anesthetic vaporizer (useful for extended surgeries) or drop method[28] (used only for short procedures, typically less than 5 min). These inhalational anesthesia methods have the advantage of providing more stable physiologic

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parameters (for example, heart rate, respiratory rate, oxygen saturation) than does hypothermia in neonatal rats or mice,[16] although only a few studies have evaluated their behavioral effects.[18,24,27]

To date, few published studies have investigated the effects of different types of neonatal anesthesia on research outcomes and animal welfare.[11,16,18,37] This knowledge gap leaves researchers uninformed of potential anesthetic effects on research outcomes and hampers the ability of veterinary professionals to provide appropriate guidance to scientists working with these animals. These considerations indicate a critical need to perform assessments of wellbeing for neonatal rodents undergoing anesthesia. Outcome measures used in prior research on this topic in adult rodents include behavioral measures, including defecation, urination, rearing, vocalization,[19] grimacing,[20,31] nest building,[13,25] and grooming,[25] as well as blood biomarkers including norepinephrine,[15,32,34] corticosterone,[15,32,34] and the neutrophil:lymphocyte ratio.[32-34] Evidence on the reliability and validity of these parameters as measures of stress is sparse and varied in neonatal rodents,[3,16,32] in part due to their developmental immaturity, altriciality, and small blood volume.

A promising, noninvasive approach to evaluating stress in response to anesthesia in both younger and older neonatal rats is by measuring ultrasonic vocalizations (USV). Neonatal rats emit USV in the 40- to 70-kHz range, with 2 distinct classes of USV: 40 kHz and 66 kHz.[3] Both classes of USV are observed in aversive situations, such as rough handling by the dam and controlled foot shock delivery, and are considered to indicate distress and a negative affective state of the neonatal rat.[3] More invasive measures of stress and pain have been studied in adults or neonates, including blood biomarkers such as corticosterone,[15,16,36] norepinephrine,[15] and glucose.[15] However, because the blood volume of young neonatal rats is so small, euthanasia of the animal typically is necessary to yield a sufficient volume of blood for testing. This requirement is suboptimal, not only with regard to the animal welfare goal of reducing the number of animals used but also because it limits the implementation of more complex study designs (for example, studies of stress using repeated measures).

In the present study, we investigated the acute response of neonatal rats of various postnatal ages to multiple anesthetic methods using the approach of evaluating USV and serum concentrations of norepinephrine, corticosterone, and glucose. We hypothesized that these outcomes would provide insight into the wellbeing of the manipulated neonatal rats and that animals undergoing inhalational anesthesia (isoflurane or sevoflurane) would experience less distress than those undergoing hypothermia anesthesia.

Materials and Methods

Animals. Male and female Sprague–Dawley rat pups (267 males and 269 females; *Rattus norvegicus*; Crl:CD(SD) IGS; Charles River, Raleigh, NC) generated from healthy, unmanipulated adult rats bred inhouse (48 litters) were used in the current study. Breeding males were separated from dams at birth, and all pups (range, 4 to 16 per litter) remained in their dam's cage until used in the experiments. All pups in a litter were tested on the same day. Date of birth was defined as postnatal day 0 (PND 0). Male and female adults were paired again after use of the pups. Rats were free of pinworms (*Syphacia muris*, *S. obvelata*, *Aspiculuris tetraptera*), fur mites (*Myocoptes*, *Radfordia*, *Myobia*), rat coronavirus, rat Theiler virus, Kilham rat virus, rat parvovirus, Toolan H1 virus, rat minute virus, Sendai virus, pneumonia virus of mice, and *Mycoplasma pulmonis*, as monitored by soiled-bedding sentinels and quarterly through exhaust air dust testing.

Rats were housed in IVC (21.3 cm [high] × 34.6 cm [long] × 39.6 cm [wide]; GR900, Tecniplast, Buguggiate, Italy) on corncob bedding (1/4 in., irradiated, Bed-O-Cobs, Lab Supply, Durham, NC) with red tunnel tubes (K3325, Red, Certified, Rat Tunnel, Bio-Serv, Flemington, NJ) as enrichment and brown crinkle paper (EnviroPak, Lab Supply) as nesting material in a room with a 12:12-h light:dark cycle (lights on, 0700; lights off, 1900) at 70 to 74 °F (21.1 to 23.3 °C), and 30 to 70% relative humidity. Rats were provided food (5V0F, Select Rodent 50 IF/6F Auto, Lab Diet, St Louis, MO) and reverse osmosis–filtered water ad libitum. All experiments were approved by the University of North Carolina at Chapel Hill IACUC. All rats were treated in accordance with the *Guide for the Care and Use of Laboratory Animals*[17] in an AAALAC-accredited facility.

Experimental design. Prior to the start of experimental manipulation, the dam and litter were gently moved from their home cage to a clean static mouse cage without bedding for baseline USV collection of the dam with the litter. After baseline recording, the dam and pups were returned to their home cage. Pups were then used in groups of as many as 4 for experimental group assignments and recording of preanesthesia vocalization (T0). Prior to manipulation of pups in the home cage (removing or returning pups), the dam was removed from the home cage and placed temporarily in a clean cage. When pups were returned to the home cage, they were placed in the nest alongside the unmanipulated litter mates and covered with nesting material to encourage maternal acceptance. The dam was returned to the cage after any manipulation of pups (removal or return). When not being weighed or in the USV recording chamber, including during maintenance of anesthesia and while recovering, pups that had been removed from their dam were maintained in a clean, empty cage on top of a heating pad. Pups for each age group (PND 2, 5, 8, 11, and 14) were assigned the next consecutive number that was assigned randomly by using Excel (Microsoft, Redmond, WA) to groups according to age and sex. We tested 5 types of anesthesia: hypothermia (PND 2, 5, and 8 only, in accordance with the *AVMA Guidelines*[21]), isoflurane by vaporizer, sevoflurane by vaporizer, isoflurane by the drop method, sevoflurane by the drop method[28]. We also included 2 controls: a baseline control group (that is, no manipulation) and a no-anesthesia control group (that is, pups were removed from the dam and placed in a clean cage on a heating pad to experience maternal separation but did not undergo anesthesia). A power analysis determined that a group size of 14 pups was sufficient to detect a typical effect size and variance, power of 0.80, and an α level of 0.05. Each group contained at least 7 pups of each sex, with any differences in group size due to using all pups from a single litter on a particular day (Table 1).

Pups in the baseline control group were sexed, weighed, and then swiftly decapitated for blood collection. Pups that were used for recording vocalizations and anesthesia were sexed, weighed, and identified by using a nontoxic permanent marker to create a unique marking on the feet or tail. Pups were then placed into individual USV recording chambers for preanesthesia recording (T0), after which they were returned to their home cage. After all pups in the litter had been recorded, pups were removed in groups (maximum, 4 pups per group) for anesthesia; pups were returned to the dam after recovery from anesthesia. USV recordings were repeated at 10 and 120 min after recovery from anesthesia (T10 and T120, respectively). Prior to being placed in the recording chamber at T120, all rats

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Table 1. Demographics of experimental animals used

	Baseline	No Anesthesia	Hypothermia	Isoflurane (drop method)	Isoflurane (vaporizer)	Sevoflurane (drop method)	Sevoflurane (vaporizer)	Total
	n = 97	n = 77	n = 53	n = 82	n = 74	n = 83	n = 70	n = 536
Postnatal day								
2	19	16	16	17	14	16	14	112
5	23	16	23	15	18	15	14	124
8	17	15	14	15	14	16	14	105
11	17	16	NA	17	14	17	14	95
14	21	14	NA	18	14	19	14	100
Age group								
Younger	59	47	53	47	46	47	42	341
Older	38	30	NA	35	28	36	28	195
Sex								
Female	48	39	27	42	36	42	35	269
Male	49	38	26	40	38	41	35	267

NA, not applicable; older, PND 11 and 14; younger, PND 2, 5, and 8.

were inspected for any visible evidence of injury related to toe pinch or maternal aggression. Immediately after T120 USV recording, pups were swiftly decapitated, and trunk blood was collected into an anticoagulant-free serum separator tube. The total volume of blood collected (50 to 600 µL) differed according to the size of the pup. Tubes were centrifuged (2,000 × g × 10 min), and serum was placed in cryovials at –80 °C until biomarker analysis. The experimental design depicting each experimental group is presented in Figure 1.

Anesthesia. Pups remained with dam until induction of anesthesia. They were removed from the dam in groups (maximum, 4 pups) and were anesthetized by using their preassigned method. Groups were selected to include only 1 of each anesthesia group, because of space and equipment limitations. For all anesthetic groups, time at induction (start of anesthesia), time of surgical plane of anesthesia (defined as loss of the pedal withdrawal reflex), time at end of anesthesia, and time of recovery (defined as return of the righting reflex) were assessed and recorded. Pedal withdrawal reflex was tested by using a consistent, firm pinch to the digits on each of the 4 feet, with loss of the reflex defined as no response (including paw withdrawal or increase of visually assessed respiratory rate) to toe pinch on all 4 feet. Pedal withdrawal was assessed during the induction phase of anesthesia after loss of righting reflex, and assessment was repeated approximately every 30 s as often as necessary until complete loss of response to toe pinch was achieved. To limit variability, the same person, an experienced veterinarian, performed all procedures, including testing of the pedal withdrawal reflex. To scavenge waste anesthetic gas, all anesthesia was performed in a biosafety cabinet. During anesthesia, all rats were continuously monitored visually for changes in respiratory rate and character.

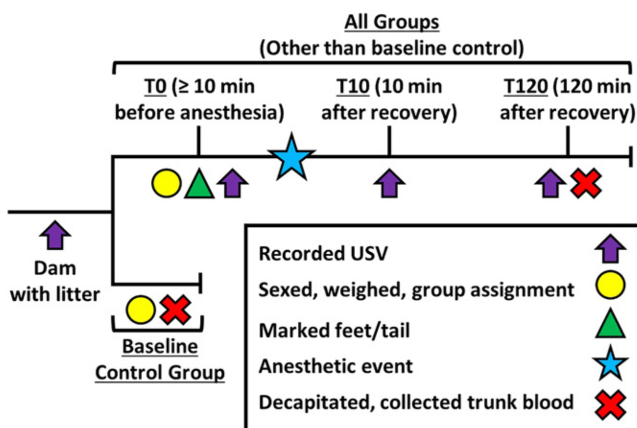

Figure 1. Experimental design. For each litter, pups and dam underwent baseline USV recording. Pups were sexed, weighed, and randomly assigned to an experimental group (maximum, 4 per group). Pups in baseline control group were decapitated and blood collected. Pups in other experimental groups were identified, then individual baseline USV recorded at T0 (at least 10 min before start of anesthesia). Pups were then anesthetized according to their anesthesia group or underwent maternal separation only (no-anesthesia group). After anesthetic recovery or maternal separation, USV were recorded at T10 and T120. After T120 USV recording, pups were decapitated and blood collected. See Table 1 for numbers of animals in each group.

Anesthesia time, defined as loss of pedal withdrawal reflex to end of anesthesia delivery, was maintained for 10 min for the hypothermia and vaporizer groups. For the hypothermia group, anesthesia was performed as previously described.[15] Briefly, pups were wrapped in a piece of latex, placed in an ice bath and held in position until anesthesia was attained; pups were then held in the ice water in the same position for the total duration of the anesthesia time (10 min), ensuring that pups' paws were maintained above the surface of the ice water to remain accessible for testing pedal withdrawal reflex. To mitigate potential tissue damage or discomfort, pups were not permitted to come into direct contact with the ice or water.

For the groups that received isoflurane (IsoSol, VEDCo, St. Joseph, MO) and sevoflurane (SevoFlo, Zoetis, Parsippany, NJ) by vaporizer, anesthesia was induced by using an induction chamber (1 L/min 100% O_2 containing isoflurane at 5% or sevoflurane at 8%) until the animal lost its righting reflex, at which time it was transferred to an appropriately sized rodent nose cone until loss of pedal withdrawal reflex, after which it was maintained (1 L/min of 100% O_2 with isoflurane at 1 to 2.5% or sevoflurane at 3 to 5%). The ranges of concentrations of inhalants used for maintenance of anesthesia were selected according to 0.7 to 1.5 times the minimum alveolar concentration of each anesthetic, with anesthesia most commonly being maintained in the middle of the range (1.0 to 1.25 times the minimum alveolar concentration).[24,34] All rats were observed during anesthesia, and anesthetic concentration was adjusted as needed for maintaining a surgical plane while avoiding a deep plane (decreased respiratory rate and increased respiratory effort).

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For the groups that received isoflurane and sevoflurane by the drop anesthesia method, a 14- or 50-mL conical tube (depending on the size of the pup's head) was precharged with less than one-quarter capful of anesthetic (approximately 0.5 mL) that was added to a piece of gauze in the bottom of the conical tube. The pup was held by scruffing and positioned at the opening of the conical tube until anesthesia was induced. Anesthesia was maintained for 2 min by keeping the pup's nose within the opening of the conical tube, allowing mixture of room air to avoid death from overdose.

After anesthesia, pups were placed in a clean cage on a heating pad (medium temperature setting, Mikikin, China) for recovery. After return of the righting reflex and once pups were ambulatory (or, for PND 2 and 5 pups, consistently moving limbs and crawling forward), they were returned to their home cage and monitored closely until maternal acceptance was confirmed.

USV recordings. ***Recording chambers.*** We used 4 sound-attenuating chambers to record USV, similar to methods previously described.[5,30] Briefly, we drilled a small hole in the top of each chamber (a hard-sided cooler with internal dimensions of 27 cm [length] × 23 cm [width] × 47 cm [height]) to allow the microphone cord to exit. The ultrasonic microphone (UltraSoundGate CM16/CMPA, Avisoft Biocoustics, Glienicke, Germany) was located inside the recording chamber, centered 35 cm above the chamber bottom, and the recording device (UltraSoundGate 116Hb, Avisoft Biocoustics) was outside the chamber. The recording software (RECORDER USGH, Avisoft Biocoustics) was configured with a sampling rate of 250,000 Hz, fast Fourier transform (FFT) length of 256 points, time-window overlap of 50%, flat top weighted window, and 16-bit format. All recordings were completed in a quiet room.

USV recording sessions. USV recordings were performed similarly to methods previously described.[3,5,30] For baseline recording, the dam and litter of pups were moved from their home cage to a clean static mouse cage free from bedding. The animals were placed in the testing chamber and allowed to acclimate to the closed chamber for 10 min. A 5-min baseline USV recording was then collected, and the rats were returned to their home cage.

In addition, USV recordings were obtained from each pup at T0 (at least 10 min before the start of anesthesia) and at 10 and 120 min after recovery (T10 and T120, respectively). For these recordings, each pup was placed individually into an empty static mouse cage (or, for PND 2 and 5 pups, a clean, empty, open culture dish) in the recording chamber. A 5-min USV recording was then immediately collected, and each pup was returned to its home cage. The recording chamber was cleaned with Peroxigard (Peroxigard, Oakville, Ontario, Canada) and allowed to dry between pups.

Quantification of USV. The 5-minute USV recordings yielded spectrograms that were analyzed similarly to methods previously described.[3,5,10] Briefly, recordings were transferred to Avisoft-SASLab Pro (version 5.3.00, Avisoft Bioacoustics) for analysis. FFT was used to generate spectrograms with parameters of FFT length of 256, temporal resolution overlap of 75%, 100% frame size, and flat top weighted window. Acoustic signals were detected via the program's automatic whistle tracking algorithm, with a threshold of –20 decibels, a minimum duration of 3 ms, and a hold time of 2 ms. These parameters were wide to allow detection of small calls; and an investigator who was blinded to the treatment group and trained in USV identification verified the accuracy of detection. Individual USV were analyzed for duration (in s), mean frequency (in kHz), and total number of vocalizations during the 5-min period.

Serum neuroendocrine biomarkers. ***Serum corticosterone.*** Serum corticosterone was measured by using a corticosterone rat/mouse ELISA kit (07DE-9922, MP Biomedicals, Solon, OH). Serum samples were undiluted. The plates were read on an ELISA plate reader set to 450 nm by using SoftMax Pro 7.0 (Molecular Devices, Sunnyvale, CA). Concentrations were calculated by using the 4-parameter logistic curve assay on MyAssays.com. Values were returned as 'low' when lower than 10 ng/mL and were set to 0 in the analyses (n = 27 of 541 samples).

Serum norepinephrine. Serum norepinephrine was measured by using a norepinephrine ELISA kit (LS-F5550, LifeSpan BioSciences, Seattle, WA). The majority of the samples were undiluted, but occasionally we had insufficient serum for the 50 µL of sample required to perform the test. For these samples, the serum was diluted 1:1 with the diluent provided with the kit, and the values were adjusted after reading. The plates were read on an ELISA plate reader set to 450 nm by using SoftMax Pro 7.0 (Molecular Devices). Concentrations were calculated using the 4-parameter logistic curve assay on MyAssays.com. In our approach, values would have been returned as 'low' when lower than 45 pg/mL and as 'high' when greater than 5000 pg/mL, but none of the values observed in our study crossed these thresholds.

Serum blood glucose. Serum blood glucose was measured by using a glucometer (CareSens N Blood Glucose Monitoring System, iSens, Torrance, CA). Values were returned as 'low' when lower than 20 mg/dL (n = 113 of 537 samples) or deemed 'insufficient' when the blood volume was too small to obtain a valid reading (n = 4 of 537 samples). Low values were set to 0, and insufficient readings were excluded from analysis.

Statistical analysis. The design described yielded a nested (that is, nonindependent) data structure. For the USV outcomes, repeated USV measures at T0, T10, and T120 (level 1) were nested within animal (level 2), and animal nested within litter (level 3). For the blood biomarkers, animal (level 1) was nested within litter (level 2). Accordingly, either a 3- or 2-level mixed-effects modeling approach was used to examine the effect of PND age, biologic sex, baseline vocalization (USV outcomes only), and experimental condition, both in unadjusted and adjusted models. Apart from USV count and glucose, all models were linear mixed models, with the intercept treated as random and all other predictors treated as fixed. USV count and glucose concentration were modeled by using a generalized estimating equation with a Poisson distribution. The USV count outcome was divided by 100 to facilitate model estimation and convergence. Analyses were stratified by PND age—younger (PND 2, 5, and 8) or older (PND 11 and 14) in the USV models. Stata 15 (StataCorp LLC, College Station, TX) was used to complete the analyses. For all analyses, a P value less than 0.05 was considered significant.

Results

Inclusion in study and demographics. Overall, 536 rat pups were included in the final analytic sample (Table 1). Six rats died due to anesthetic overdose or failure to recover from anesthesia (5 pups that received isoflurane by drop [1 each from PND 5 and 8, and 3 from PND 14] and 1 PND 2 pup that received sevoflurane by drop) and were excluded from all analyses. We found no significant difference in the weights of rats used at each PND when compared between anesthesia groups, although weights differed significantly in a predictable pattern when compared

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between PND; younger pups on average weighed less than older pups ($P < 0.0001$; Table S1). χ^2 testing to determine whether the distribution of rats by PND was equivalent across conditions did not reveal a statistically significant difference within either the younger (PND 2, 5, and 8; $\chi^2_{12} = 2.72$, $P = 0.997$) or older (PND 11 and 14; $\chi^2_5 = 0.57$, $P = 0.989$) age groups.

Maternal acceptance. Each time pups were returned to the dam (that is, after either anesthesia or USV collection), the animals were observed for maternal acceptance or adverse treatment (for example, rough maternal handling, maternal rejection) of the pups. Throughout the study, all dams accepted and cared for the pups that were returned to their cage. None of the pups had visible evidence of tissue-related injury from maternal aggression or other procedures (for example, skin damage from hypothermia, toe pinch) throughout the study.

Times to surgical plane of anesthesia and recovery. The time to surgical plane of anesthesia (that is, loss of the pedal withdrawal reflex) was significantly ($P \leq 0.001$) shorter for all inhalational anesthesia and age groups as compared with the hypothermia group (Figure 2A). The time to recovery from anesthesia (that is, return of the righting reflex) was significantly ($P \leq 0.001$) shorter in all inhalational anesthesia and age groups as compared with the hypothermia group (Figure 2 B). We found no significant differences in time to surgical plane of anesthesia or recovery with regard to age or anesthesia group other than hypothermia.

USV. Table 2 and Figure 3 summarize the mean frequency, duration, and total number of USV divided by 100 in a 5-min period. Specifically, Table 2 provides descriptive statistics (mean ± 1 SD) of overall USV from each anesthesia and PND age group. At T10 min after recovery, the mean duration of USV was significantly ($P \leq 0.05$) greater in the hypothermia group than in the group that received isoflurane by vaporizer (Figure 3C). At T10 and T120, the mean total number of USV produced by the hypothermia group was significantly ($P \leq 0.05$) less than those produced by all other groups (Figure 3E). Overall, the nature of the USV changed as PND increased. Across all PND age groups, regardless of anesthesia group, older rat pups produced USV at a lower frequency, longer duration, and a higher total number than did younger pups (Table 2). Interaction testing of PND age by experimental condition within each of the stratified age models showed only sporadic significant effects, with no clear pattern of systematic differences in the effect of anesthesia by PND age (Tables S2 and S3).

Serum neuroendocrine biomarkers. At PND 2, 5, and 8, serum corticosterone was significantly ($P \leq 0.001$) higher in the hypothermia group as compared with all inhalational anesthesia and control groups (Figure 4). Regardless of anesthesia type, serum corticosterone and glucose were both significantly higher in younger pups compared with the older groups, but we detected no significant differences with regard to anesthesia or PND for serum norepinephrine (Table 3). No significant differences were detected between biologic sexes for any of the neuroendocrine markers. Interaction testing of PND age by experimental condition within each of the stratified age models showed only sporadic significant effects, with no clear pattern of systematic differences in the effect of anesthesia by PND age (Tables S4 and S5).

Discussion

Overall, our data showed that USV and corticosterone were proxy indicators of distress in neonatal rats that underwent anesthesia. As compared with those in all other inhalational anesthetic approaches, pups in the hypothermia group experienced significantly longer duration of USV and higher serum corticosterone levels, suggesting that they experienced heightened distress. Furthermore, rats in the hypothermia group showed significantly longer times for induction and recovery than did pups in the other anesthesia groups; the longer duration likely increases stress relative to time spent from the dam. Therefore, according to our comprehensive outcome measures, our results strongly suggest that, among the methods of anesthesia tested, hypothermia is the least preferred method for neonatal rats.

Hypothermia has long been considered the 'gold standard' for neonatal anesthesia,[8] partially because neonatal rats are neurologically immature, with afferent pain pathways not well-developed until after PND 5 to 7.[21] Since a study in the late 1900s,[8] only one other study has investigated the effects of anesthesia on neonatal rats;[16] that study focused largely on physiologic parameters of rats while under anesthesia (for example, heart rate, respiratory rate, oxygen saturation) and noted that isoflurane or sevoflurane anesthesia allowed better control of these parameters as compared with hypothermia anesthesia. In contrast to our current study, the previous study[16] did not find significant differences in corticosterone between anesthesia groups. However, we found that corticosterone was significantly higher in rats undergoing hypothermia anesthesia as compared with all other groups. Past studies have used data showing that hypothermia anesthesia in neonatal rats does not alter growth or cognition later in life as evidence of the safety of hypothermia anesthsia.[18] Although this finding is important, it does not

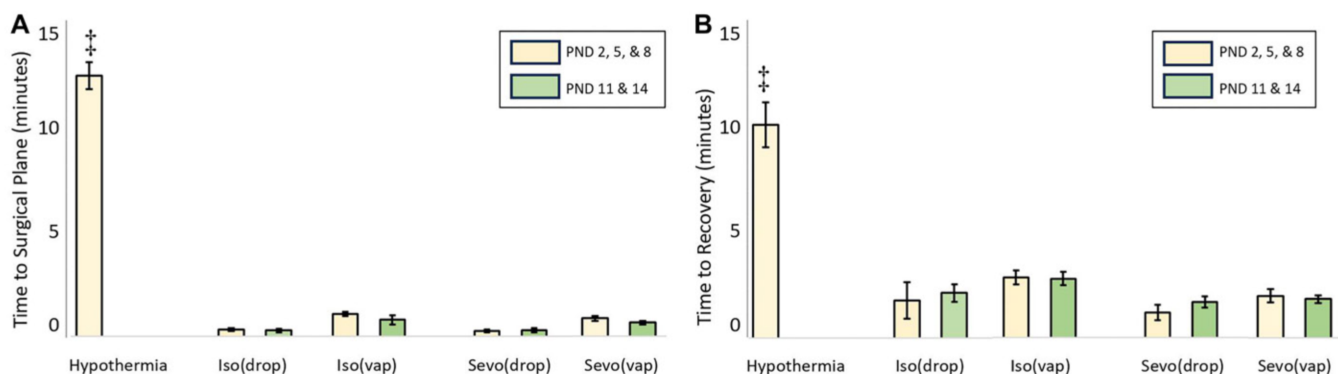

Figure 2. Times to (A) surgical plane of anesthesia (loss of the pedal withdrawal reflex) and (B) recovery from anesthesia (return of the righting reflex). Data are shown as mean ± 1 SD. ‡, Value is significantly ($P \leq 0.001$) different from that of other groups among younger neonates (PND 2, 5, and 8); iso(drop), isoflurane administered by drop method; iso(vap), isoflurane administered by vaporizer; sevo(drop), sevoflurane administered by drop method; sevo(vap), sevoflurane administered by vaporizer. See Table 1 for numbers of animals in each group.

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Table 2. Descriptive statistics of USV outcome measures (mean ± 1 SD) across experimental groups and time points

	No Anesthesia	Hypothermia	Isoflurane (drop method)	Isoflurane (vaporizer)	Sevoflurane (drop method)	Sevoflurane (vaporizer)	Overall
Mean frequency (kHz)							
PND 2	61.6 ± 8.8	65.4 ± 11.4	62.4 ± 9.0	63.6 ± 11.3	62.8 ± 11.4	64.4 ± 9.6	63.3 ± 10.3
PND 5	48.7 ± 4.1	48.9 ± 7.9	50.2 ± 5.3	47.9 ± 3.5	49.9 ± 6.1	47.8 ± 3.8	48.9 ± 5.6
PND 8	41.8 ± 4.0	42.7 ± 5.9	42.6 ± 3.0	43.6 ± 6.3	40.9 ± 3.3	41.6 ± 3.5	42.2 ± 4.5
PND 11	41.6 ± 3.9	NA	41.0 ± 3.2	40.9 ± 3.4	43.1 ± 3.6	43.2 ± 4.6	41.9 ± 3.8
PND 14	43.6 ± 5.1	NA	43.4 ± 6.7	45.0 ± 6.5	42.8 ± 4.5	46.0 ± 7.4	44.4 ± 6.1
Younger	50.7 ± 10.2	52.1 ± 12.4	52.2 ± 10.5	51.3 ± 11.1	51.1 ± 11.9	51.2 ± 11.5	51.5 ± 11.3[a]
Older	42.5 ± 4.6	NA	42.2 ± 5.4	42.9 ± 5.5	42.9 ± 4.1	44.6 ± 6.3	43.0 ± 5.2[a]
Overall	47.5 ± 9.4	52.1 ± 12.4	48.0 ± 10.0	48.2 ± 10.3	47.6 ± 10.2	48.5 ± 10.2	48.4 ± 10.4
Duration (s)							
PND 2	0.019 ± 0.006	0.016 ± 0.008	0.019 ± 0.008	0.015 ± 0.007	0.021 ± 0.009	0.018 ± 0.005	0.018 ± 0.008
PND 5	0.029 ± 0.008	0.028 ± 0.010	0.027 ± 0.011	0.028 ± 0.009	0.027 ± 0.008	0.028 ± 0.008	0.028 ± 0.009
PND 8	0.035 ± 0.010	0.040 ± 0.017	0.032 ± 0.010	0.032 ± 0.011	0.034 ± 0.011	0.032 ± 0.009	0.034 ± 0.012
PND 11	0.037 ± 0.010	NA	0.042 ± 0.014	0.037 ± 0.013	0.034 ± 0.009	0.041 ± 0.015	0.038 ± 0.013
PND 14	0.040 ± 0.020	NA	0.038 ± 0.016	0.040 ± 0.013	0.039 ± 0.013	0.035 ± 0.012	0.039 ± 0.015
Younger	0.027 ± 0.010	0.028 ± 0.015[c]	0.026 ± 0.011	0.025 ± 0.011[c]	0.028 ± 0.011	0.026 ± 0.009	0.027 ± 0.011[b]
Older	0.039 ± 0.015	NA	0.040 ± 0.015	0.039 ± 0.013	0.037 ± 0.012	0.038 ± 0.014	0.038 ± 0.014[b]
Overall	0.032 ± 0.0135	0.028 ± 0.015	0.032 ± 0.014	0.030 ± 0.014	0.032 ± 0.012	0.031 ± 0.013	0.031 ± 0.014
Total no. (in 100 s)							
PND 2	5.02 ± 4.51	1.94 ± 2.12	3.74 ± 3.10	2.84 ± 3.33	4.97 ± 3.71	3.29 ± 3.15	3.66 ± 3.55
PND 5	6.56 ± 4.28	5.64 ± 4.14	6.27 ± 4.09	6.31 ± 4.22	6.32 ± 4.78	6.23 ± 4.20	6.18 ± 4.26
PND 8	4.76 ± 4.00	3.42 ± 3.56	5.75 ± 4.26	5.41 ± 4.70	6.22 ± 5.20	7.15 ± 6.29	5.46 ± 4.84
PND 11	13.76 ± 7.80	NA	9.81 ± 7.40	8.28 ± 7.72	13.20 ± 9.93	7.28 ± 4.65	10.63 ± 8.13
PND 14	5.13 ± 5.17	NA	5.84 ± 5.67	5.03 ± 5.63	6.36 ± 4.47	6.57 ± 4.92	5.83 ± 5.17
Younger	5.46 ± 4.32[d]	3.93 ± 3.82[d]	5.19 ± 3.95[d]	4.98 ± 4.36[d]	5.82 ± 4.61[d]	5.56 ± 4.98[d]	5.13 ± 4.37
Older	9.79 ± 7.95	NA	7.77 ± 6.83	6.66 ± 6.91	9.59 ± 8.27	6.92 ± 4.77	8.22 ± 7.21
Overall	7.13 ± 6.34	3.93 ± 3.82	6.29 ± 5.51	5.61 ± 5.51	7.46 ± 6.71	6.10 ± 4.93	6.23 ± 5.74

NA, not applicable, older, postnatal days 11 and 14; overall, all groups or time points combined; Younger, postnatal days 2, 5, and 8.
[a]Value significantly ($P < 0.001$) different for mean frequency of USV between younger and older neonates.
[b]Value significantly ($P < 0.05$) different for duration of USV between younger and older neonates.
[c]Value significantly ($P < 0.05$) different for mean duration of USV between hypothermia and isoflurane (vaporizer) groups in younger neonates.
[d]Value significantly ($P < 0.05$) different for total number of USV in 5 min between hypothermia and all other groups in younger neonates.

address the acute levels of distress or pain experienced by the neonatal rats undergoing hypothermia anesthesia.

In the current study, we examined USV in neonatal rats, which are known to exhibit USV in the 40- to 70-kHz range when exposed to aversive situations, such as when isolated from the dam.[3] We showed that the USV of rat pups change significantly as they age. Specifically, among all PND age groups from 2 to 14 d, as age increased, rat pups produced USV of lower frequency, longer duration, and a higher total number as compared with younger pups, regardless of anesthesia group. At the 10-min recovery time point after anesthesia, the hypothermia group experienced significantly longer duration of and fewer total number of USV than did the other groups. This pattern was present in all age groups. These findings, coupled with the elevated serum corticosterone in the hypothermia group, suggest that the longer duration of each call correlates with a heightened state of distress. Given that the use of USV to assess animal distress is relatively new, these results have important implications for the interpretation of USV data from neonatal rats. More studies are needed to assess USV as an outcome measure for the assessment of neonatal rat wellbeing during anesthesia.

From an animal welfare perspective, inhalational anesthetics were objectively easier to use than hypothermia, as demonstrated by the significantly shorter times to induction and recovery, consistent with previous reports.[16] This conclusion contradicts conventional wisdom that cites ease of use as a benefit of hypothermia anesthesia.[16,18] The longer time spent away from the dam for pups undergoing hypothermia anesthesia may further exacerbate distress due to maternal separation, as measured by USV.[3] Inhalational anesthetic methods require more financial resources than hypothermia, given that they include purchasing the anesthetic gas and may require a vaporizer. For drop methods of anesthesia, a fume hood or hard-ducted biosafety cabinet must be used to scavenge waste anesthetic gasses[17] and therefore may be less convenient. The few deaths that occurred during our current study were only in pups that had undergone a drop method of anesthesia; however, we saw only 6 deaths overall with the drop method (3.6% of 165 rats anesthetized with the drop method), and these deaths were spread across the PND age groups. Vaporizer methods allowed titration of the anesthetic to avoid overdose, whereas drop methods offer limited control over the dose provided. Investigators should consider that vaporizer methods provide a more stable and reproducible plane of anesthesia and should be used rather than the drop method for longer periods of anesthesia. Waste gas from

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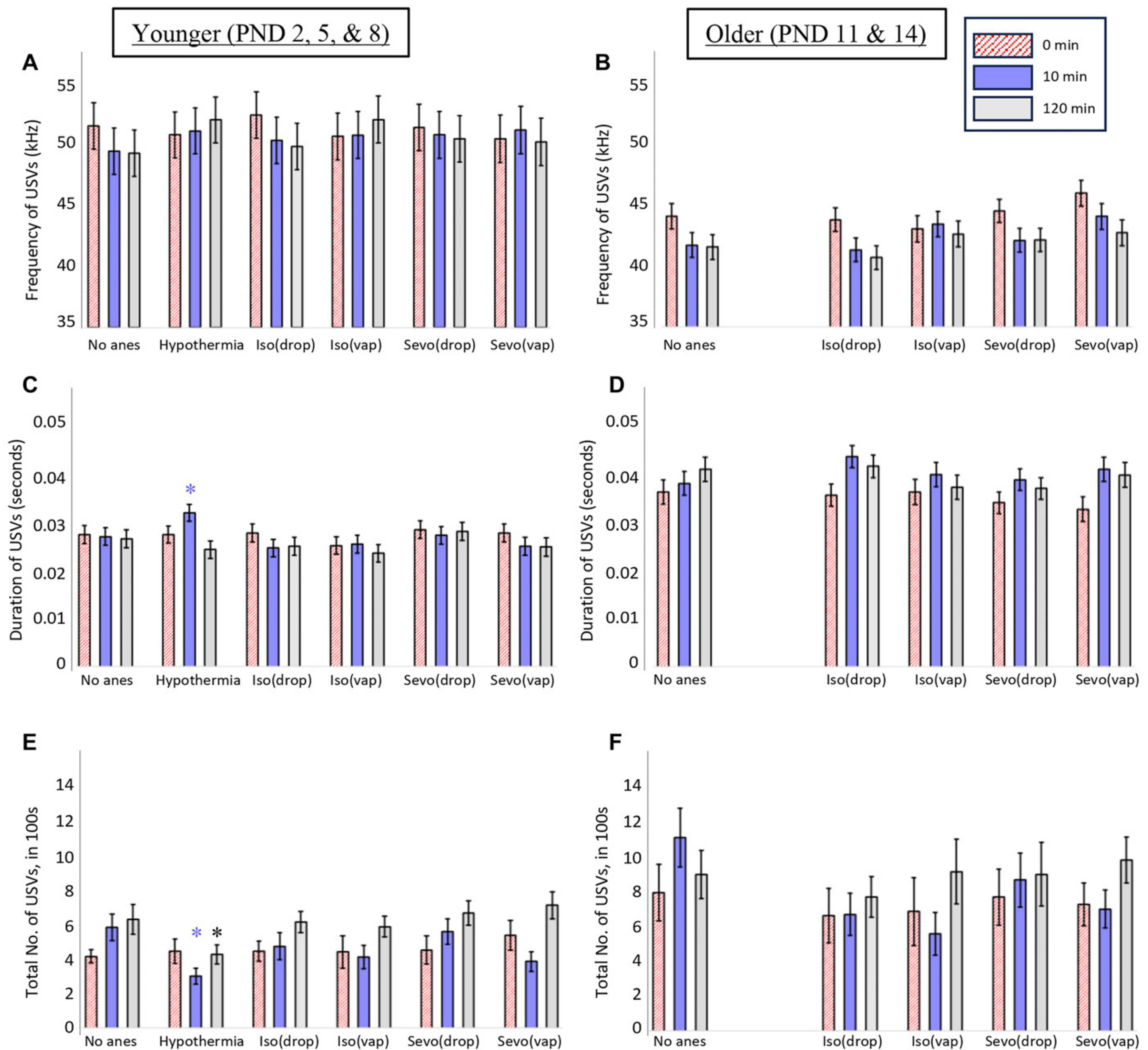

Figure 3. USV data at time points before and after anesthesia. Left panels show data from PND 2, 5, and 8 pups; right panels show data from PND 11 and 14 pups. Mean frequency of USV for (A) PND 2, 5, and 8 pups and (B) for PND 11 and 14 groups. Mean duration of USV for (C) PND 2, 5, and 8 groups and (D) PND 11 and 14 pups. Mean of total number of USV in 100 s (E) for PND 2, 5, and 8 groups and (F) for PND 11 and 14 rats. Data are shown as mean ± 1 SD. *, Value is significantly ($P \leq 0.05$) different from that for other groups at the same time point; no anes, no-anesthesia group; iso(drop), isoflurane administered by drop method; iso(vap), isoflurane administered by vaporizer; sevo(drop), sevoflurane administered by drop method; sevo(vap), sevoflurane administered by vaporizer. See Table 1 for numbers of animals used in each group.

inhalational anesthetics can pose an occupational health risk,[23] which—although minimized when appropriate environmental, health, and safety guidelines are followed—are not an issue when using hypothermia anesthesia. Furthermore, performing anesthesia for neonatal rats in a biosafety cabinet, as we did here, may cause excess cooling of the neonates due to the rapid movement of air in the cabinet. We used a heating pad to provide warmth for neonatal rats in our study, but a limitation of our study is that the air movement in the biosafety cabinet may have substantially cooled the neonatal rats during anesthesia. Future studies could monitor the body temperature of the neonatal rats during anesthesia to ensure adequate provision of heat. Given these considerations, we conclude that the greatest benefits of hypothermia anesthesia are that it is less costly and requires less infrastructure than do other methods. However, its use has multiple animal welfare and study design drawbacks, chief among them being greater animal stress, more time needed for anesthesia induction and recovery, and potential confounding of study interpretation because anesthetized animals experience some degree of stress or pain.

In addition to corticosterone, this study investigated norepinephrine and glucose as potential biomarkers of stress. These biomarkers are potential indicators of the acute stress response due to their relationship with the sympathetic–adrenal–medullar system and hypothalamic–pituitary–adrenal axis.[15] We found no significant differences in norepinephrine or glucose levels between anesthesia groups, possibly indicating that these compounds are not sufficiently sensitive measures of stress in

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Table 3. Serum corticosterone, norepinephrine, and glucose values (mean ± 1 SD) across experimental conditions

	Baseline	No Anesthesia	Hypothermia	Isoflurane (drop method)	Isoflurane (vaporizer)	Sevoflurane (drop method)	Sevoflurane (vaporizer)	Overall
Corticosterone (ng/mL)								
PND 2	158.9 ± 54.5[a]	190.4 ± 85.1[a]	231.2 ± 107.8	154.9 ± 53.5[a]	198.6 ± 84.0[a]	174.7 ± 54.0[a]	196.8 ± 70.0[a]	188.5 ± 79.8
PND 5	58.7 ± 44.0[a]	79.7 ± 34.9[a]	127.6 ± 59.0	87.1 ± 51.8[a]	87.0 ± 41.6[a]	65.7 ± 33.6[a]	78.5 ± 32.3[a]	86.7 ± 48.7
PND 8	74.4 ± 42.5[a]	63.0 ± 26.9[a]	156.6 ± 58.1	83.4 ± 56.7[a]	52.4 ± 37.4[a]	52.9 ± 47.1[a]	59.3 ± 35.0[a]	77.1 ± 56.6
PND 11	54.5 ± 27.9	56.0 ± 28.8	NA	62.1 ± 29.0	58.5 ± 32.6	51.1 ± 25.8	61.4 ± 38.9	57.5 ± 30.8
PND 14	79.0 ± 44.5	63.5 ± 25.1	NA	76.3 ± 27.3	37.2 ± 31.2	70.6 ± 34.8	76.5 ± 31.8	66.9 ± 34.4
Younger	98.1 ± 63.4[a]	115.7 ± 82.2[a]	170.9 ± 89.9	112.1 ± 63.4[a]	114.0 ± 86.8[a]	98.5 ± 71.6[a]	111.5 ± 78.0[a]	119.5 ± 81.8
Older	68.0 ± 39.5[c]	59.5 ± 27.3[c]	NA	69.4 ± 28.9[c]	47.9 ± 33.5[b,c]	61.4 ± 32.3[c]	69.0 ± 36.1[c]	62.2 ± 33.0[c]
Overall	83.9 ± 56.8	92.2 ± 70.4	170.9 ± 89.9	93.0 ± 55.4	86.8 ± 76.2	82.4 ± 60.7	94.5 ± 67.8	97.7 ± 72.8
Norepinephrine (pg/mL)								
PND 2	301.3 ± 150.7	294.8 ± 131.7	334.8 ± 132.3	362.2 ± 152.1	300.2 ± 204.3	259.7 ± 152.8	320.9 ± 159.5	312.1 ± 156.9
PND 5	365.5 ± 212.8	268.5 ± 141.2	227.0 ± 149.9	302.9 ± 189.0	298.2 ± 108.5	365.9 ± 151.3	432.6 ± 180.9	318.0 ± 172.4
PND 8	382.3 ± 247.5	384.8 ± 165.1	336.3 ± 127.9	432.0 ± 315.4	348.0 ± 169.7	390.7 ± 173.1	348.8 ± 121.1	374.9 ± 194.1
PND 11	317.7 ± 122.7	326.1 ± 126.5	NA	384.3 ± 166.7	398.2 ± 133.3	296.4 ± 101.7	400.0 ± 106.4	357.4 ± 134.7
PND 14	326.3 ± 184.8	264.3 ± 160.5	NA	301.3 ± 134.35	330.4 ± 215.3	328.5 ± 132.9	302.4 ± 175.2	307.7 ± 163.6
Younger	351.2 ± 207.1[b]	322.3 ± 154.0	296.3 ± 147.4	370.1 ± 231.8	318.6 ± 165.2	337.6 ± 168.8	367.4 ± 161.8	336.4 ± 177.22
Older	322.5 ± 158.1	296.2 ± 146.4	NA	341.5 ± 155.8	366.9 ± 178.0	314.3 ± 120.7	351.2 ± 152.2	332.0 ± 152.0
Overall	339.3 ± 188.2	309.6 ± 150.9	296.3 ± 147.4	357.0 ± 201.7	335.7 ± 171.5	327.7 ± 150.4	360.9 ± 157.8	333.6 ± 168.1
Glucose (mg/dL)								
PND 2	79.3 ± 78.9	128.6 ± 86.5	115.5 ± 93.2	110.9 ± 91.2	97.9 ± 100.1	85.8 ± 100.8	65.1 ± 87.0	100.2 ± 93.8
PND 5	144.0 ± 62.9	180.1 ± 58.5	140.5 ± 83.6	145.1 ± 86.2	162.9 ± 78.0	176.1 ± 68.6	152.1 ± 95.1	157.2 ± 79.2
PND 8	116.9 ± 114.1	136.5 ± 108.5	158.9 ± 104.5	143.1 ± 115.7	115.1 ± 110.9	128.3 ± 115.0	164.5 ± 105.6	139.3 ± 110.7
PND 11	214.0 ± 60.6	234.2 ± 29.8	NA	227.1 ± 67.6	228.9 ± 16.9	237.8 ± 24.4	223.1 ± 12.1	229.4 ± 39.3
PND 14	205.2 ± 96.2	225.4 ± 66.5	NA	237.7 ± 61.2	241.8 ± 13.5	233.7 ± 59.4	242.7 ± 21.8	233.7 ± 56.4
Younger	112.5 ± 89.2	144.6 ± 91.2	137.5 ± 94.3	131.2 ± 99.45	122.7 ± 100.3	128.0 ± 103.7	128.8 ± 105.3	130.4 ± 98.6
Older	209.2 ± 81.3[d]	230.1 ± 50.3[d]	NA	232.6 ± 64.3[d]	235.3 ± 16.5[d]	235.7 ± 46.2[d]	232.9 ± 20.1[d]	231.6 ± 48.7[d]
Overall	152.2 ± 97.1	181.1 ± 86.7	137.5 ± 94.3	175.5 ± 99.2	169.3 ± 95.2	175.3 ± 99.1	171.0 ± 96.7	169.1 ± 96.3

NA, not applicable; older, postnatal days 11 and 14; overall, all groups or time points combined; younger, postnatal day 2, 5, and 8.
[a]Value significantly ($P < 0.001$) different compared with hypothermia.
[b]Value significantly ($P < 0.05$) different compared with hypothermia.
[c]Value significantly ($P < 0.05$) different compared with younger neonates.
[d]Value significantly ($P < 0.001$) different compared with younger neonates.

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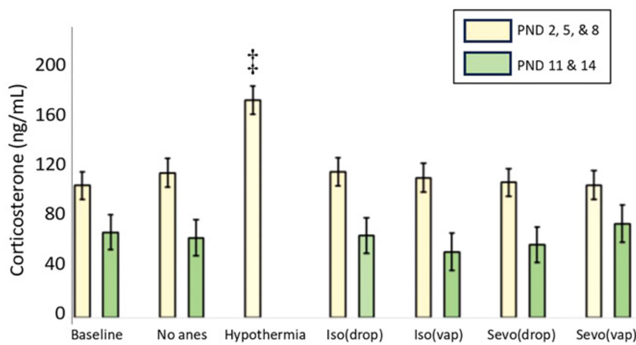

Figure 4. Serum corticosterone values at baseline or 120 min after anesthesia. Data are shown as mean ± 1 SD. ‡, Value is significantly ($P \leq 0.001$) different from that for other groups within PND 2, 5, and 8; no anes, no-anesthesia group; iso(drop), isoflurane administered by drop method; iso(vap), isoflurane administered by vaporizer; sevo(drop), sevoflurane administered by drop method; sevo (vap), sevoflurane administered by vaporizer. See Table 1 for numbers of animals used in each group.

this context. Alternatively, a single anesthetic event at these ages may not be an adequately strong stressor to cause acute changes to these biomarkers. Additional research is needed to better understand whether and how norepinephrine and glucose might be markers of stress in neonatal rats. An additional finding was that both serum corticosterone and glucose were significantly higher in younger as compared with older neonatal rats; this difference is likely due to developmental changes as the neonates age and was a consideration in including multiple PND ages in this study and in treating age as a covariate in the adjusted model results.

Some limitations of this study were related to the detection of background noise and associated requirements for successful USV behavioral testing. The USV of the dam and pups together, which were recorded prior to the study, were not used in the analyses for this study, because we had no way to distinguish between overlapping vocalizations of the dam and multiple pups. Future studies should consider recording baseline data with the dam and a single pup to better observe differences in the USV of the pup with and without the dam present. USV were not collected during induction of anesthesia or during recovery. Due to a limited recording chamber size and challenges associated with providing a consistent, reliable heat source in the sound-attenuated chamber, the neonates had no external heat source during USV recording. Lack of a heat source in the USV chamber may have falsely elevated USV as cold temperatures are a cause of USV production in neonates.[3] However, because we used control pups to account for this potential issue, we believe that the overall findings and conclusions are valid.

The primary purpose of this study was to assess USV and neuroendocrine marker outcomes from neonates undergoing different types of anesthesia; therefore, we cannot speculate on how the results might differ if neonates underwent a noninvasive or invasive procedure as part of a standard experimental protocol. Experimental procedures may potentially alter recovery times and overall results. Given that surgical procedures tend to generally increase animal pain and distress, we speculate that the use of a more stressful anesthesia like hypothermia would only further heighten animal stress, and this question merits further study. We did not investigate the use of injectable anesthetics, such as pentobarbital, due to their unpredictable availability and typical high cost, which limit the likelihood that researchers will choose this agent. However, alternative injectable anesthetics could be evaluated in future studies. Another limitation is that we focused on reaching a surgical plane of anesthesia, which was defined as loss of the pedal withdrawal reflex, as previously described.[15] However, we did not evaluate physiologic parameters, such as heart rate and respiratory rate. Future studies could include assessments of physiologic parameters to monitor the rats more completely during anesthesia.

Collectively, the novel USV outcome measures should be further evaluated as a tool for quantifying other distressful or painful stimuli such as methods for the induction of pain. The current study focused on mean frequency, duration, and number of USV produced, rather than on the structural features of the call patterns. Future studies could investigate characteristics of the acoustic structure[3,4] of rodent USV. Finally, future studies could assess the chronic effects of neonatal anesthesia on later research outcomes.

Overall, USV and corticosterone appeared to be useful as proxy indicators of wellbeing in neonatal rats undergoing anesthesia. Veterinarians and investigators performing anesthesia of neonatal rats for future studies should consider that, according to our data, the inhalational anesthetic methods tested here (isoflurane and sevoflurane via vaporizer and drop method) are preferable to hypothermia anesthesia. This recommendation is a departure from current guidance and has the potential to greatly benefit animal wellbeing.

Supplemental Material

Table S1. Mean Weights of Experimental Animals in Grams

Table S2. Multilevel models predicting USV for younger neonatal rats, $N = 282$

Table S3. Multilevel models predicting USV for older neonatal rats, $N = 157$

Table S4. Multilevel models predicting neuroendocrine markers for younger neonatal rats, $N = 314$

Table S5. Multilevel models predicting neuroendocrine markers for older neonatal rats, $N = 195$

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