ANESTHESIOLOGY

Disruption of Rapid Eye Movement Sleep Homeostasis in Adolescent Rats after Neonatal Anesthesia

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ANESTHESIOLOGY 2019; 130:981–94

ABSTRACT

Background: Previous studies suggest that rapid eye movement sleep rebound and disruption of rapid eye movement sleep architecture occur during the first 24 h after general anesthesia with volatile anesthetics in adult rats. However, it is unknown whether rapid eye movement sleep alterations persist beyond the anesthetic recovery phase in neonatal rats. This study tested the hypothesis that rapid eye movement sleep disturbances would be present in adolescent rats treated with anesthesia on postnatal day 7.

Methods: Forty-four neonatal rats were randomly allocated to treatment with anesthesia consisting of midazolam, nitrous oxide, and isoflurane or control conditions for 2 h or 6 h. Electroencephalographic and electromyographic electrodes were implanted and recordings obtained between postnatal days 26 and 34. The primary outcome was time spent in rapid eye movement sleep. Data were analyzed using two-tailed unpaired *t* tests and two-way repeated measures analysis of variance.

Results: Rats treated with midazolam, nitrous oxide, and isoflurane exhibited a significant increase in rapid eye movement sleep three weeks later when compared with control rats, regardless of whether they were treated for 2 h (174.0 ± 7.2 min in anesthetized, 108.6 ± 5.3 in controls, $P < 0.0001$) or 6 h (151.6 ± 9.9 min in anesthetized, 108.8 ± 7.1 in controls, $P = 0.002$).

Conclusions: Treatment with midazolam, nitrous oxide, and isoflurane on postnatal day 7 increases rapid eye movement sleep three weeks later in rats.

(ANESTHESIOLOGY 2019; 130:981–94)

EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Anesthesia in adult rodents has been associated with alterations in sleep architecture that persist up to 18 h after anesthesia

What This Article Tells Us That Is New

- Anesthesia with isoflurane, nitrous oxide, and midazolam on postnatal day 7 is associated with alterations in sleep architecture three weeks later in adolescent rats

Numerous laboratories have documented widespread neuronal degeneration and persistent learning deficits in a variety of developing species after exposure to common inhaled and intravenous anesthetics.[1–7] Several retrospective clinical studies, especially of longer and repeated exposures, have also linked childhood anesthesia and surgery to cognitive impairment.[8–11] However, the clinical significance of these findings remains unclear, because of concerns regarding the translational value of the animal models used in bench research and the confounding factors associated with retrospective studies in humans. Although several clinical trials are ongoing,[12,13] the results of three recent large-scale studies (General Anesthesia and Awake-Regional Anesthesia in Infancy [GAS],[14] Pediatric Anesthesia Neurodevelopment Assessment [PANDA],[15] and Mayo Anesthesia Safety in Kids [MASK][16]) suggest that anesthetic exposures of less than 1 h do not lead to severe cognitive deficits in young children. Although these clinical data are consistent with most animal studies that find little evidence for neurodevelopmental effects after brief exposures, it remains unclear whether longer anesthetics are safe.

To date, most preclinical and clinical studies of anesthetic-related neurotoxicity have focused on learning disabilities as the primary outcome, whereas very few have investigated the effects of anesthetic drugs on other functional, noncognitive domains.[17,18] However, several lines of evidence support the idea that anesthetic drugs interfere with endogenous sleep pathways and can affect sleep–wake behavior in the short term. For example, adult rats exposed to 6 h of isoflurane exhibit lower levels of slow wave sleep in

Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org). Portions of this work were presented previously at the Association of University Anesthesiologists 2018 Annual Meeting, April 26–27, Chicago, Illinois, and the International Anesthesia Research Society 2018 Annual Meeting, April 28–May 1, Chicago, Illinois.

Submitted for publication February 18, 2018. Accepted for publication January 30, 2019. Corrected on March 22, 2019. From the Department of Anesthesiology (N.L., N.A., C.K., Z.Z.) and Department of Neurology (H.P.G.), University of Virginia Health System, Charlottesville, Virginia; School of Medicine (R.S.), Department of Pharmacology and Neuroscience Graduate Program (K.A.S.), and Department of Pharmacology (M.P.B.), University of Virginia, Charlottesville, Virginia.

<zdoi;10.1097/ALN.0000000000002660>

the 18 h after general anesthesia, compared with sham-controls.[19] Also, rapid eye movement sleep debt accrues in adult mice exposed to 6 h of isoflurane or sevoflurane immediately after anesthesia.[20] Importantly, it is becoming increasingly evident that sleep is crucial for the assembly of new synaptic circuits during brain development, learning, and memory. For example, Boyce *et al.*[21] recently demonstrated that optogenetic silencing of neurons activated during rapid eye movement sleep after learning impairs fear-conditioned contextual memory (a test of hippocampus-dependent learning and memory), establishing direct causality between rapid eye movement sleep and memory consolidation. Li *et al.*[22] also recently showed that pruning of new dendritic spines is impaired during development and motor learning in rapid eye movement sleep–deprived mice.

Thus, the aim of this study was to determine whether exposure to anesthetic drugs during a phase of intense brain plasticity interferes with subsequent sleep–wake behavior. We examined the effects of a combination of anesthetics on the sleep–wake behavior of adolescent rats after a single brief (2h) exposure on postnatal day 7 (Anesthesia 2h group). In keeping with previous studies from our laboratory that documented decreased synaptic density and disruption of presynaptic vesicle spatial organization after prolonged anesthetics,[2,3] we also assessed the effects of a 6h exposure to the same anesthetic protocol (Anesthesia 6h group). Our specific hypothesis was that rapid eye movement sleep disturbance would be present in adolescent rats exposed to anesthesia at neonatal age. The primary outcome was time spent in rapid eye movement sleep.

Materials and Methods

Animals and Anesthesia

We exposed Sprague–Dawley rat pups to anesthesia at postnatal day 7. Postnatal day 0 was considered the day of birth. Experimental rats were exposed to either 2 h (Anesthesia 2h group, N = 10) or 6 h (Anesthesia 6h group, N = 11) of anesthesia; sham-controls were exposed to 6 h of sham anesthesia (N = 11 sham-controls in Anesthesia 2h group, N = 12 sham-controls in Anesthesia 6h group). An approximately equal number of male and female rats were used (mean weight: 16.03 g, Envigo, USA). Pups from nine different litters were used to control for litter variability. Animals were allocated to experimental and sham-control groups the morning of anesthesia using the random Excel function. Experimental animals received intraperitoneal midazolam (9 mg/kg, Sigma-Aldrich Chemical, USA) freshly dissolved in dimethyl sulfoxide at a final concentration of 0.1% and were placed in a clear plexiglass anesthesia chamber kept inside a temperature chamber set at 37 ± 1°C (Hotpack, USA). Nitrous oxide (70%), isoflurane (0.75%), and oxygen (30%) were delivered into the anesthesia chamber by a calibrated flowmeter. Isoflurane was administered using an isoflurane-specific calibrated vaporizer. Animals were continuously observed during anesthesia and the inspired gas concentrations monitored with an inline Datex Capnomac Ultima gas analyzer (Datex Ohmeda, USA). Sham-control animals received an equal volume of vehicle (0.1% dimethyl sulfoxide) intraperitoneally and were kept in a separate chamber at room air. Temperature inside the chamber was maintained at 37 ± 1°C *via* a heating pad. At the conclusion of anesthesia, pups were recovered until return of spontaneous movements and reunited with their mothers. In general, the animals tolerated the anesthetic exposure well.

Of note, midazolam 9 mg/kg does not cause loss of righting reflex (the behavioral endpoint of sedation) in rodents[23,24] and the minimum alveolar concentration (MAC) that prevents movement to pain in 50% of subjects (MAC EC_{50}, the behavioral endpoint of general anesthesia) is 2.3% for isoflurane[25] and 175% for nitrous oxide[26,27] in rats. In line with this, and as shown in table 1, rats in the Anesthesia 2h group remained sedated through the 2h exposure (righting reflex scores of 1 and toe pinch scores above 1). Anesthesia 6h rats were sedated during the first 4 h of exposure and achieved a surgical plane of anesthesia during the last 2 h (scores of 1 for both righting reflex and toe pinch).

A priori power calculations were based on two-tailed *t* test of mean difference using preliminary data of total time spent in rapid eye movement sleep in a 4h period in sham-control and anesthesia-exposed rats. We determined that a group size of n = 22 (11 rats per group) would provide 80% power to detect a 30% change in rapid eye movement sleep with the expected mean ± SD at 9 (3%). All studies were approved by the Institutional Animal Care and Use Committee at the University of Virginia (Charlottesville, Virginia) and conducted in accordance with the National Institutes of Health (Bethesda, Maryland) guidelines. Figure 1 is a schematic representation of the experimental design.

Assessment of Anesthesia Depth and Physiologic Monitoring

The level of anesthesia depth was assessed by toe pinch and righting reflex scores at baseline (0 h) and at 1, 2, 3, 4, 5 and 6 h of anesthetic exposure (table 1). A toe pinch test was performed first and the response was scored on a scale

Table 1. Monitoring of Anesthesia Depth

Time Point (h)	Toe Pinch Score (1–5)	Righting Reflex Score (1–5)
0	5 (5-5)	5 (5-5)
1	4 (4-3)	1 (1-1)
2	3 (3-2)	1 (1-1)
3	2 (2-2)	1 (1-1)
4	2 (2-1)	1 (1-1)
5	1 (1-1)	1 (1-1)

Toe pinch and righting reflex score presented as median with interquartile range.

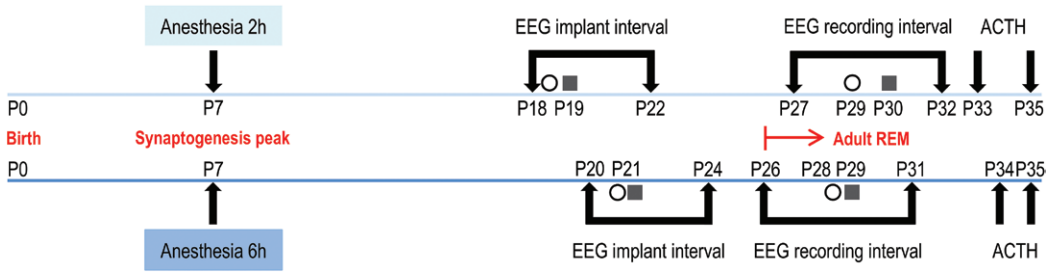

Fig. 1. Schematic representation of the experimental design. Median age of electroencephalographic (EEG) implant and EEG recording is indicated for sham-controls (○ symbol) and anesthesia-exposed (■ symbol) rats for both Anesthesia 2h and Anesthesia 6h groups. (*Anesthesia 2h group*: Sham-controls: median EEG implant: postnatal day [P]18 median EEG recording: P29; Anesthesia-exposed: median EEG implant: P19, median EEG recording: P30. *Anesthesia 6h group*: Sham-controls: median EEG implant: P21, median EEG recording: P28.5; Anesthesia-exposed: median EEG implant: 21, median EEG recording: P29). ACTH, adrenocorticotropic hormone.

from 1 to 5 as follows: 1 = *no spontaneous activity, no response to toe pinch*; 2 = *no spontaneous activity, single limb withdrawal to pinch*; 3 = *no spontaneous activity, purposeful movement to toe pinch*; 4 = *moving spontaneously but slowly and irregularly*; and 5 = *fully awake, moving regularly and spontaneously*. The righting reflex was then evaluated by placing the infant rat in the supine position and observing whether and how it was able to turn prone. The righting reflex was scored on a scale from 1 to 5 as follows: 1 = *complete loss of righting reflex, no response to being placed in supine position*; 2 = *uncoordinated movements and unable to return to prone position in 30 s*; 3 = *uncoordinated movements initially but able to return to prone position in 30 s*; 4 = *coordinated movements, returns to prone position in less than 10 s*; and 5 = *awake, returns to prone position immediately*. All depth of anesthesia assessments were performed by the same investigator. In addition, heart rate (HR), oxygen peripheral saturation (SpO_2), and respiratory rate (RR) were monitored at baseline (0 h) and at 1, 2, 3, 4, 5 and 6 h of anesthetic exposure (see fig. 2). HR and SpO_2 were measured using a PhysioSuite monitor (Kent Scientific, USA). RR was determined *via* observation of abdominal movements for 30 s at each of the indicated time points. All RR measurements were performed by the same investigator.

Surgery

Rats were surgically implanted with four electroencephalographic and one electromyographic electrodes for recording of sleep–wake states between postnatal days 18 to 24 (see fig. 1). Briefly, anesthesia was induced with 2.5 to 3% isoflurane and maintained with 1.5 to 2% isoflurane and 35% oxygen. Rats were placed into a stereotactic frame (Stoelting, USA) and kept at a temperature of 37 ± 1°C *via* a heating pad. Blunt ear bars were used to stabilize the animals' head. The skin over the scalp was shaved and scrubbed with alcohol and povidone–iodine solution. A midline incision was performed to expose the skull and permit the drilling of four Burr holes (approximately 0.6 mm in

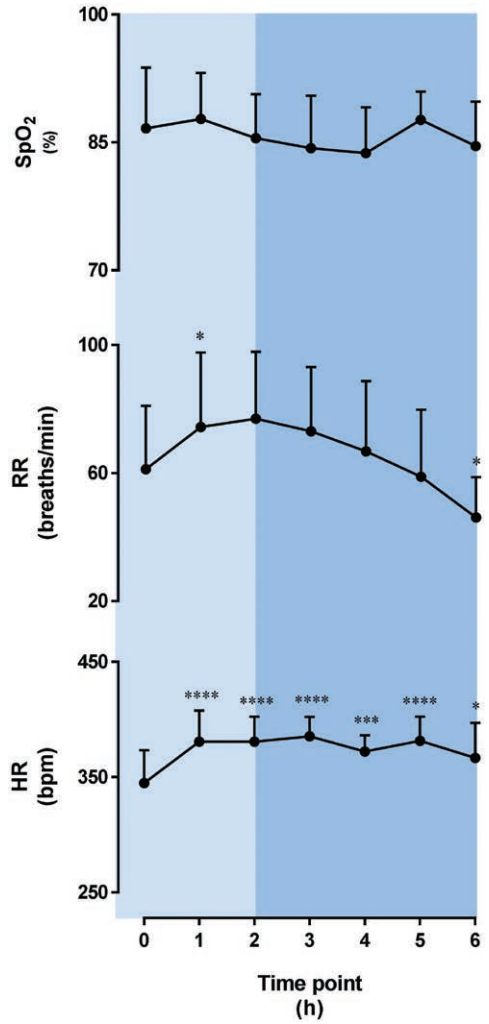

Fig. 2. Physiologic monitoring during anesthetic exposure. Two-way repeated measures ANOVA (*$P < 0.05$; ***$P < 0.001$; ****$P < 0.0001$). HR, heart rate; RR, respiratory rate; SpO_2, oxygen peripheral saturation.

diameter) to accommodate four epidural electrodes (left and right frontal cortex, and left and right parietal cortex, respectively), with an additional hole drilled over the right cerebellum (to accommodate a sinus ground electrode). Electrodes consisted of stainless steel screws affixed with stainless steel Teflon-insulated wire. In addition, one insulated stainless steel wire was sutured to the dorsal neck muscles to record nuchal electromyographic. All wires were capped with stainless steel and gold-plated socket contacts and inserted into a 6-channel, light plastic pedestal secured to the skull with dental acrylic. All materials were supplied by Plastics One (USA). Postoperative analgesia was provided with ketoprofene. We are not aware of any documented effects of ketoprofene on sleep.

Electroencephalographic Recording

Electroencephalographic recordings were obtained between postnatal days 26 and 34. Median interval time between electroencephalographic implant and electroencephalographic recording was 9 days for Anesthesia 2h group and 8 days for Anesthesia 6h group (see fig. 1). Each rat was subjected to several 24h electroencephalographic/electromyographic recordings within this time interval. One 24h recording was randomly chosen per each animal for data analysis (analysis range postnatal day 26 to 32). The decision to start recording at postnatal day 26 was based on the consideration that rats attain adult levels of rapid eye movement sleep at about the end of their fourth postnatal week.[28] This age is estimated to correspond to adolescence in rats.[29] Rats were placed in an individual cage and their head stage tethered to a commutator *via* lightweight flexible cables. They were habituated to the electroencephalographic cables and cages for at least 24 h before recording. Rats were free to navigate throughout the cage and were provided food and water *ad libitum*. Animals were maintained in a dedicated electroencephalographic room on a 12h light: 12h dark cycle. Electroencephalographic and electromyographic waveforms were amplified and sampled at 200 Hertz (Hz) using a 16-channel extracellular differential amplifier (model 3500, A-M systems, USA). High- and low-pass filters were set at 0.1 Hz and 100 Hz for electroencephalograph, and 1.0 Hz and 500 Hz for electromyograph, respectively.

Data were processed through a MatLab-based application (Mathworks, USA) and LabChart software (Biopac Systems Inc., The Netherlands), and analyzed with SleepSign (Kissei, Japan) and MatLab-based software. States of rapid eye movement sleep, non–rapid eye movement sleep and wakefulness were first automatically scored by the software, then manually verified by an experienced scorer, and randomly sampled for scoring accuracy by a board certified sleep medicine physician on the basis of the predominant state within each 4s epoch. Both scorers were blinded to experimental condition. There was a 95% overall agreement between double-scored recordings of sleep–wake states. Wakefulness was scored when low-amplitude, high-frequency, desynchronized electroencephalographic activity was combined with elevated muscular tone.[20,30–32] Non–rapid eye movement sleep was scored when high-amplitude, low-frequency electroencephalographic activity was coupled with reduced motor tone compared with wakefulness.[20,30–32] Rapid eye movement sleep was scored when low-amplitude, high-frequency, synchronized electroencephalographic activity was coupled with minimal to absent muscular tone.[20,30–32] Sleep structure was evaluated by analyzing the number of bouts of each state per hour, their average duration, rapid eye movement sleep and non–rapid eye movement sleep latency, and slow wave sleep. A bout was counted as one continuous episode in a state. Average bout duration was calculated as the total time spent in a state divided by the total number of bouts in that state. Rapid eye movement sleep and non–rapid eye movement sleep latency were measured as the time elapsed from light onset until the first bout of rapid eye movement sleep and non–rapid eye movement sleep, respectively. Electroencephalographic waveforms were fast Fourier transformed in 4 s epochs. Slow wave sleep or delta power, defined as the electroencephalographic power between 0.5 and 4 Hz during non–rapid eye movement sleep, was averaged over the 24h recording period and normalized by the total power for each animal.

Plasma Corticotropin Measurement

We measured plasma adrenocorticotropic hormone in anesthesia-exposed and sham-control rats subjected to electroencephalographic implant and recording. Because blood collection was a terminal procedure (open thoracotomy and left ventricle puncture), blood was collected at the end of the last recording (postnatal days 33 to 35). Blood collection was performed between 10:00 AM and 12:00 PM for all animals. We used an enzyme-linked immunosorbent assay adrenocorticotropic hormone kit per manufacturer instructions (M046006, MD Bioproducts, USA). Briefly, whole blood was placed in EDTA tubes after collection. Plasma was immediately separated by centrifugation at 1000*g* for 20 min at 4°C and stored at −80°C for further analysis. Plasma samples were diluted 1:10. Standards and plasma samples from sham-control and anesthesia-exposed rats were simultaneously incubated with the enzyme-linked antibody and a biotin-coupled antibody in a streptavidin-coated microplate well. At the end of incubation, the microwells were washed to remove unbound components and the enzyme bound to the solid phase was incubated with the substrate tetramethylbenzidine. An acidic stopping solution was added to halt the reaction and convert the color to yellow. The intensity of the yellow color was directly proportional to the concentration of adrenocorticotropic hormone in the samples. Absorbance was read at 450 nm using an enzyme-linked immunosorbent assay microplate reader (model 680). A standard curve

was plotted using absorbance unit *versus* concentration of standards and the samples' absorbance values were interpolated on the standard curve to determine adrenocorticotropic hormone concentrations in the samples (ng/ml).

Statistical Analysis

The percent of time that animals spent asleep (non–rapid eye movement + rapid eye movement sleep) and awake was plotted for each of four time blocks (first 6 h and second 6 h of the light cycle, and first 6 h and second 6 h of the dark cycle) and analyzed with a two-way repeated measures ANOVA of sleep–wake state as a function of treatment and time. HR, SpO_2 and RR were also analyzed with two-way repeated measures ANOVA. No *post hoc* testing was conducted. The total amount of time spent in rapid eye movement sleep, non–rapid eye movement sleep and wakefulness was calculated over the 24h recording period and compared between anesthesia-treated and sham-control animals with a two-tailed unpaired Student's *t* test. Number of bouts per hour, mean bout duration, rapid eye movement sleep and non–rapid eye movement sleep latency, and normalized delta power were also compared between anesthesia-exposed and sham-control animals with a two-tailed unpaired Student's *t* test. Adrenocorticotropic hormone plasma concentrations were compared between sham-control and anesthesia-treated animals using a two-tailed unpaired Student's *t* test. Statistical analyses were performed using the software package GraphPad Prism 7.0. The data were confirmed to be consistent with a Gaussian distribution. There were no missing data in the Anesthesia 2h group of rats. In the Anesthesia 6h group, one rat developed forelimb weakness two days after surgery. Data from this rat were not included in sleep–wake state analysis. All values are shown as mean ± SD. In all cases *P* values of less than 0.05 were considered statistically significant.

Results

Raw Electroencephalographic Data of Sleep–Wake States

Figure 3 shows representative electroencephalographic/electromyographic tracings from sham-control (panel A) and anesthesia-treated (panel C and E) animals. Panels B, D, and F are raster plots of sleep–wake states as a function of time of Anesthesia 2h rats and sham-controls. The sleep–wake raster plots of Anesthesia 6h rats and their sham-controls can be found in the Figure, Supplemental Digital Content 1, http://links.lww.com/ALN/B894.

Sleep–Wake Circadian Rhythm Is Not Affected by Neonatal Anesthesia

To assess for a potential influence of early life anesthesia on the brain's circadian pacemaker, we evaluated patterns of sleep (non–rapid eye movement + rapid eye movement sleep) and wakefulness in relation to

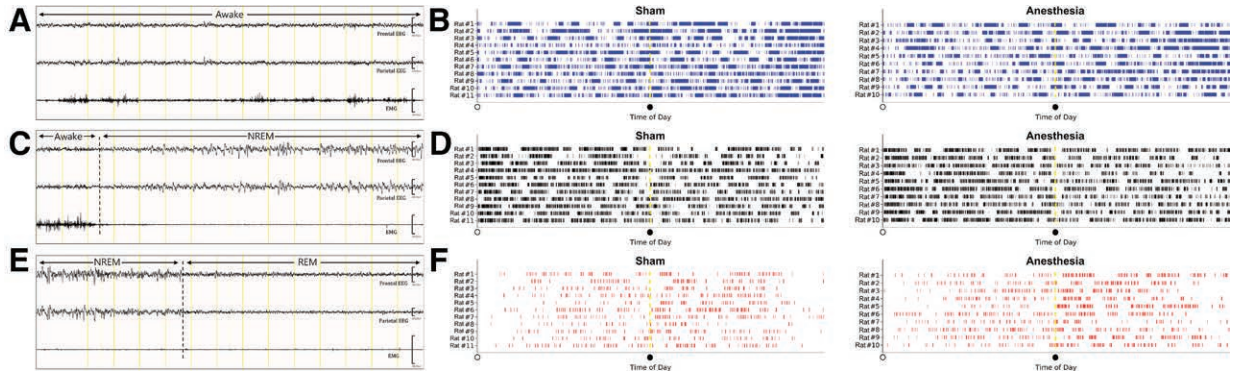

Fig. 3. Raw electroencephalogram (EEG) data of sleep-wake states. *A*, *C*, and *E* show representative EEG and electromyogram tracings. For each, the top two rows of signals represent frontal and parietal EEG, respectively, and lower row represents electromyogram. *B*, *D*, and *F*, Raster plots of sleep–wake states as a function of time for wakefulness (in blue: *B*), non–rapid eye movement (NREM) sleep (in black: *D*), and rapid eye movement (REM) sleep (in red: *F*). On the *x* axis, time of the day. On the *y* axis, animals that recordings were obtained from. Each vertical line represents a bout. Symbol ○ indicates "light on," Symbol • indicates "light off." Yellow dotted line divides the 24-h recording period in two 12-h intervals (at the time of light off). (*A*) Tracings from an awake sham-control animal. Note the low-amplitude, high-frequency, and desynchronized EEG activity combined with elevated muscular tone. (*B*) Wakefulness raster plot of sham-control and Anesthesia 2h rats. (*C*) Transition from awake to NREM sleep in an anesthesia-exposed rat. Note the change from low-amplitude, high-frequency EEG with elevated muscular tone to high-amplitude, low-frequency EEG activity with reduced motor tone. (*D*) NREM sleep raster plot of sham-control and Anesthesia 2h rats. (*E*) Transition from NREM to REM sleep in an anesthesia-treated rat. Note the change from high-amplitude, low-frequency EEG activity of NREM sleep to low-amplitude, high-frequency, synchronized EEG activity coupled with minimal to absent muscle tone of REM sleep. (*F*) REM sleep raster plots of sham-control and Anesthesia 2h animals. N = 11 sham-control and 10 Anesthesia 2h rats.

light/dark cues and compared them between anesthesia-treated and sham-control rats. Notably, rodents are nocturnal animals (*i.e.*, they are asleep when light is on and are active when light is off). As shown in figure 4A, Anesthesia 2h and sham-control animals spent the majority of time asleep when lights were on (rest phase), and progressively less time asleep after lights were turned off (active phase). Conversely, they spent less time awake when lights were on, and progressively more time awake after lights were turned off (fig. 4B). Such sleep–wake behavior reflects proper timing of rest and activity with external light cues, and thus suggests appropriate function of the internal circadian pacemaker in both sham-control and anesthesia-treated rats. Two-way repeated measures ANOVA of sleep and wake as a function of treatment and time confirmed no differences between Anesthesia 2h and sham-control rats (Treatment: $P = 0.103$; Time: $P < 0.0001$; Interaction: $P = 0.987$). Similarly, rats exposed to a 6h-long anesthesia maintained intact their ability to time rest and activity with light cues. A two-way repeated measures ANOVA of sleep and wake as a function of treatment and time showed no differences between Anesthesia 6h and sham-control rats (Treatment: $P = 0.788$; Time: $P < 0.0001$; Interaction: $P = 0.681$; see the figure, Supplemental Digital Content 2, http://links.lww.com/ALN/B895, representing patterns of sleep (non–rapid eye movement + rapid eye movement sleep) and wakefulness in Anesthesia 6h and sham-control rats).

Early-life Anesthesia Does Not Impact the Quantity or Structure of Wakefulness

The quantity and structure of wakefulness exhibited no significant changes in anesthesia-exposed adolescent rats compared with sham-controls after anesthesia administered at a young age. Figure 5A shows that the total amount of time spent in wakefulness during the 24h recording period was not significantly different in Anesthesia 2h rats compared with sham-controls. Anesthesia 2h rats were awake on average for 651.9 ± 25.0 min *versus* 728.6 ± 29.9 in sham-controls ($P = 0.067$). To assess for a potential detrimental effect of neonatal anesthesia on wakefulness structure, we analyzed the number and mean duration of wake bouts per hour. Figure 5B shows that the average number of wake bouts was not different in Anesthesia 2h rats compared with sham-controls (33 ± 2 in Anesthesia 2h rats and 34 ± 2 in sham-control rats, $P = 0.776$).

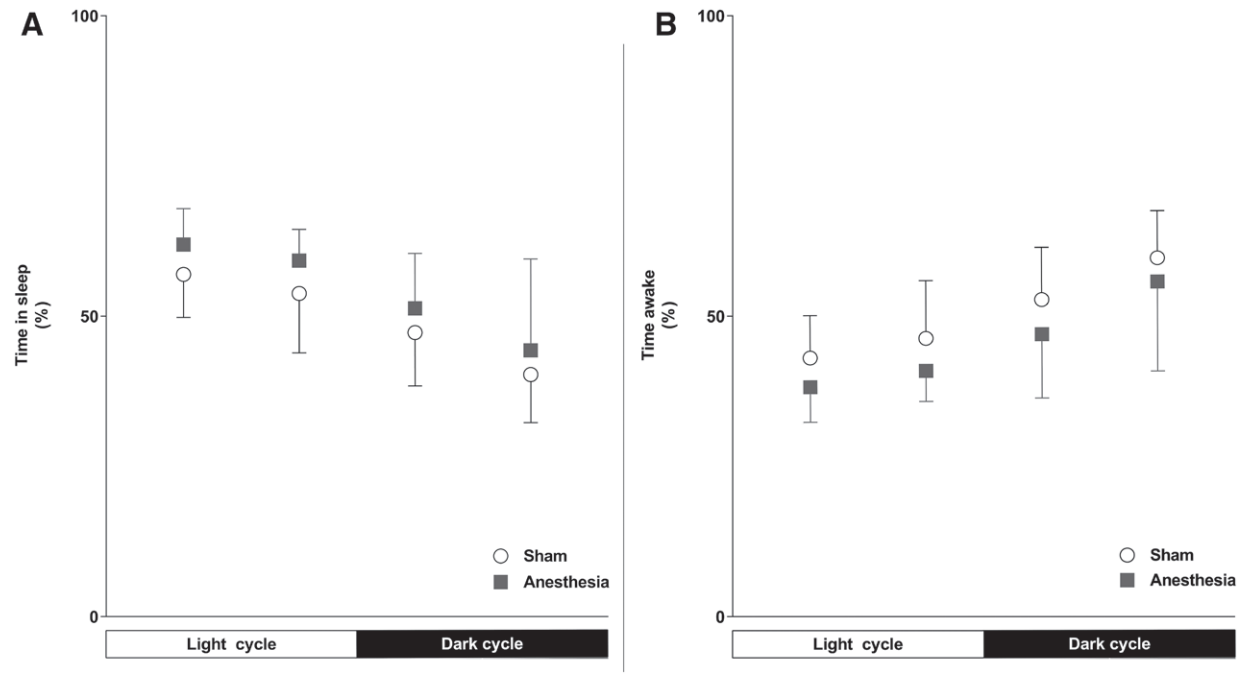

Fig. 4. Effect of neonatal anesthesia on circadian rhythm. Symbols represent mean of hourly values (n = 6 h) expressed as percent of recording time. Quiet (light) and active (dark) phase are delimited by a white and a black box, respectively, below the abscissa. (*A*) The percentage of time spent in sleep (non-rapid eye movement + rapid eye movement sleep) was appropriately high during the quiet phase and progressively lower during the active phase. A two-way repeated measures ANOVA of sleep as a function of treatment and time confirmed no effect of anesthesia on sleep (Treatment: $P = 0.103$; Time: $P < 0.0001$; Interaction: $P = 0.987$). (*B*) The percent of time spent awake was appropriately low during the quiet phase and progressively higher during the active phase. N = 11 sham-control and 10 Anesthesia 2h rats.

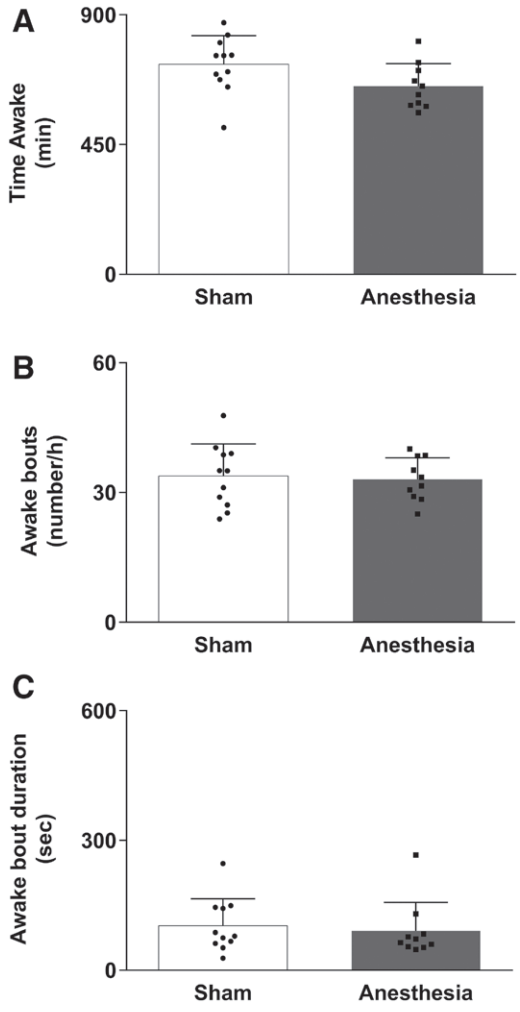

Fig. 5. Effect of neonatal anesthesia on wakefulness. (*A*) Neonatal anesthesia did not affect the total amount of time spent awake in Anesthesia 2h animals compared with sham-controls ($P = 0.067$). (*B*) The average number of wake bouts per hour was unchanged in Anesthesia 2h rats compared with sham-controls ($P = 0.776$). (*C*) The mean duration of wake bouts per hour was also unchanged in Anesthesia 2h rats compared with sham-controls ($P = 0.669$). N = 11 sham-control and 10 Anesthesia 2h rats.

Figure 5C shows that the mean duration of wake bouts was also unaffected by anesthetics. In fact, it was 90.7 ± 20.9 s in Anesthesia 2h rats *versus* 102.9 ± 18.8 in sham-control animals ($P = 0.669$). In line with the findings in the Anesthesia 2h group, there were no differences in quantity and structure of wakefulness between Anesthesia 6h and sham-control rats (see the figure, Supplemental Digital Content 3, http://links.lww.com/ALN/B896, representing total amount of time spent in wakefulness and number and mean duration of wake bouts in Anesthesia 6h rats compared with sham-controls).

Prolonged, but Not Brief, Neonatal Anesthesia Causes Increased Propensity to Enter Deep Sleep in Adolescent Rats

The quantity of non–rapid eye movement sleep of adolescent rats did not exhibit significant changes after early life anesthesia. Figure 6A shows that the total amount of time spent in non–rapid eye movement sleep during the 24h recording period was not different in Anesthesia 2h rats compared with sham-controls. Anesthesia 2h rats spent on average 614.1 ± 21.8 min in non–rapid eye movement sleep, compared with 602.8 ± 28.5 in sham-controls ($P = 0.760$). To assess for a potential detrimental effect of neonatal anesthesia on non–rapid eye movement sleep structure, we analyzed the number of non–rapid eye movement sleep bouts per hour and their mean duration, as well as latency to enter non–rapid eye movement sleep and slow wave sleep. Figure 6B shows that the number of non–rapid eye movement sleep bouts was not different between Anesthesia 2h and sham-control rats (41 ± 3 in Anesthesia 2h rats and 36 ± 2 in sham-controls; $P = 0.205$). Figure 6C shows that the mean duration of non–rapid eye movement sleep bouts was also unaffected by anesthetics. In fact, average duration of non–rapid eye movement bouts was 40.5 ± 2.6 s in Anesthesia 2h rats and 42.8 ± 1.4 in sham-controls ($P = 0.428$). When latency to enter non–rapid eye movement sleep was analyzed (fig. 6D), no difference was found in the time from light onset to first bout of non–rapid eye movement sleep in Anesthesia 2h rats compared with sham-controls (3.3 ± 0.8 min in Anesthesia 2h rats *vs.* 5.9 ± 2.1 in sham-controls, $P = 0.279$). Last, to determine the propensity to enter deep sleep we performed a Fourier transformation of the non–rapid eye movement sleep electroencephalogram and computed the power in the delta frequency range (between 0.5 and 4 Hz). As shown in figure 6E, we found no difference in delta power in Anesthesia 2h rats compared with sham controls (0.6205 ± 0.0212 in Anesthesia 2h rats *vs.* 0.6287 ± 0.0187 in sham-controls, $P = 0.775$). A significant increase in delta power was found instead in Anesthesia 6h rats compared with sham controls (0.6781 ± 0.0289 *vs.* 0.6053 ± 0.0184; $P = 0.042$; see the figure, Supplemental Digital Content 4, http://links.lww.com/ALN/B897, displaying total amount of time spent in non–rapid eye movement sleep, number and mean duration of non–rapid eye movement sleep bouts, latency to non–rapid eye movement sleep, and delta power in Anesthesia 6h rats compared with sham-controls).

Adolescent Rats Exhibit Perturbation of Rapid Eye Movement Sleep Quantity and Structure after Neonatal Anesthesia

The quantity and structure of rapid eye movement sleep were significantly disrupted in adolescent rats after neonatal exposure to anesthetic drugs. Figure 7A shows that Anesthesia 2h rats exhibited a significant 40% increase in

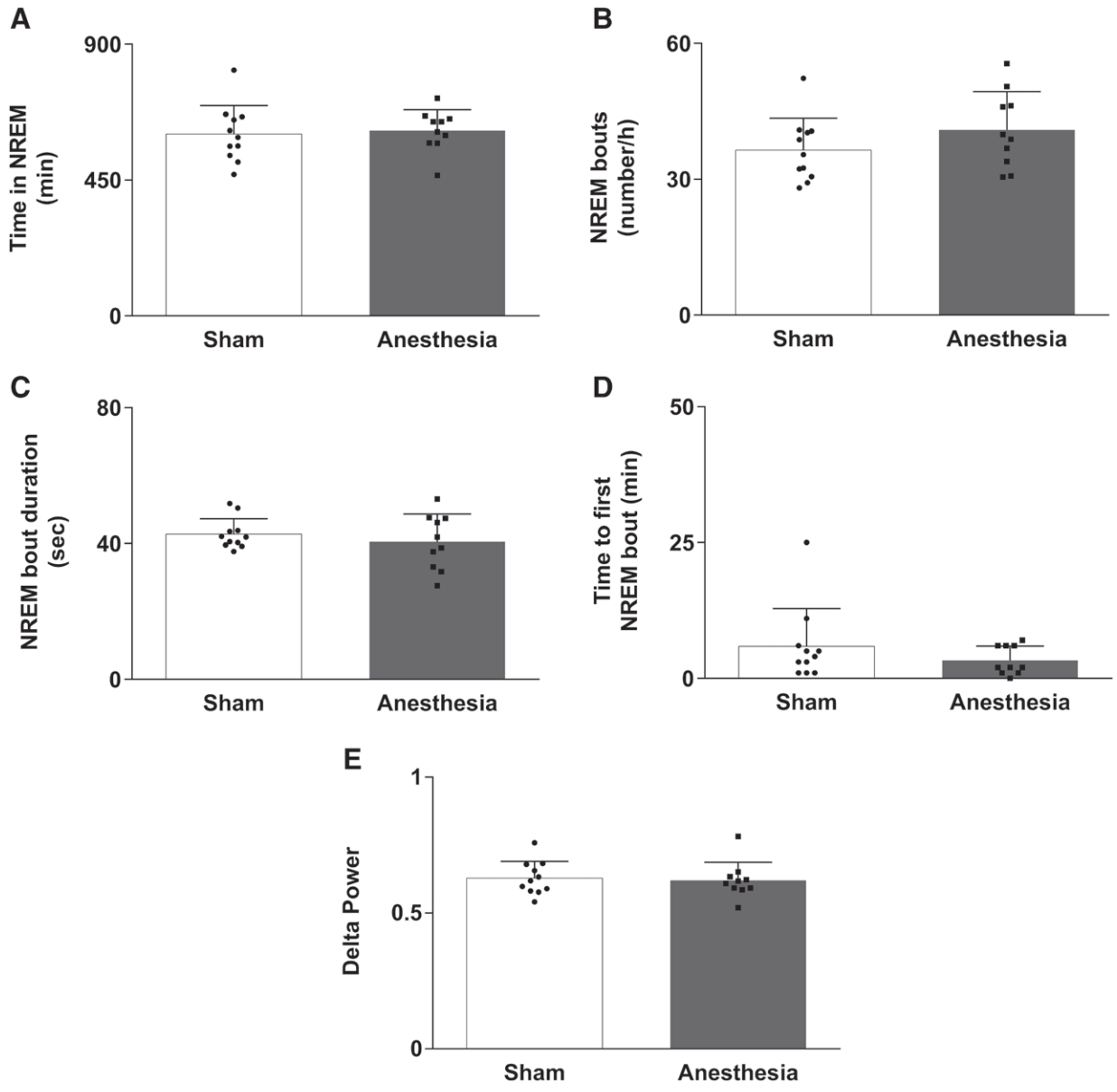

Fig. 6. Effect of early life anesthesia on non–rapid eye movement (NREM) sleep. (*A*) Early life anesthesia did not affect the total amount of time spent in NREM sleep in Anesthesia 2h animals compared with sham-controls ($P = 0.760$). (*B*) The number of NREM bouts per hour was unchanged in Anesthesia 2h rats compared with sham-controls ($P = 0.205$). (*C*) The mean duration of NREM bouts per hour was also unchanged in Anesthesia 2h rats compared with control condition ($P = 0.428$). (*D*) Latency to enter NREM sleep was not significantly changed in Anesthesia 2h rats compared with sham-controls ($P = 0.279$). (*E*) Propensity to enter deep NREM sleep (delta power) was not different in Anesthesia 2h rats compared with sham-controls ($P = 0.775$). N = 11 sham-control and 10 Anesthesia 2h rats.

the total amount of time spent in rapid eye movement sleep compared with sham-controls ($P < 0.0001$). Specifically, they spent on average 65 more minutes in rapid eye movement sleep than sham-control animals (174.0 ± 7.2 min *vs.* 108.6 ± 5.3). To assess for a potential detrimental impact of neonatal anesthesia on rapid eye movement sleep structure, we analyzed the number of rapid eye movement sleep bouts per hour and their mean duration, as well as latency to enter rapid eye movement sleep. We found that rapid eye movement sleep structure exhibited significant abnormalities that were present several days after the initial anesthetic exposure, at an age that corresponds to adolescence in rats. When the number of rapid eye movement bouts was quantified (fig. 7B), it was found to be significantly increased from 9 ± 1 in sham-controls to 17 ± 2 in Anesthesia 2h rats ($P = 0.001$). Mean duration of rapid eye movement sleep bouts

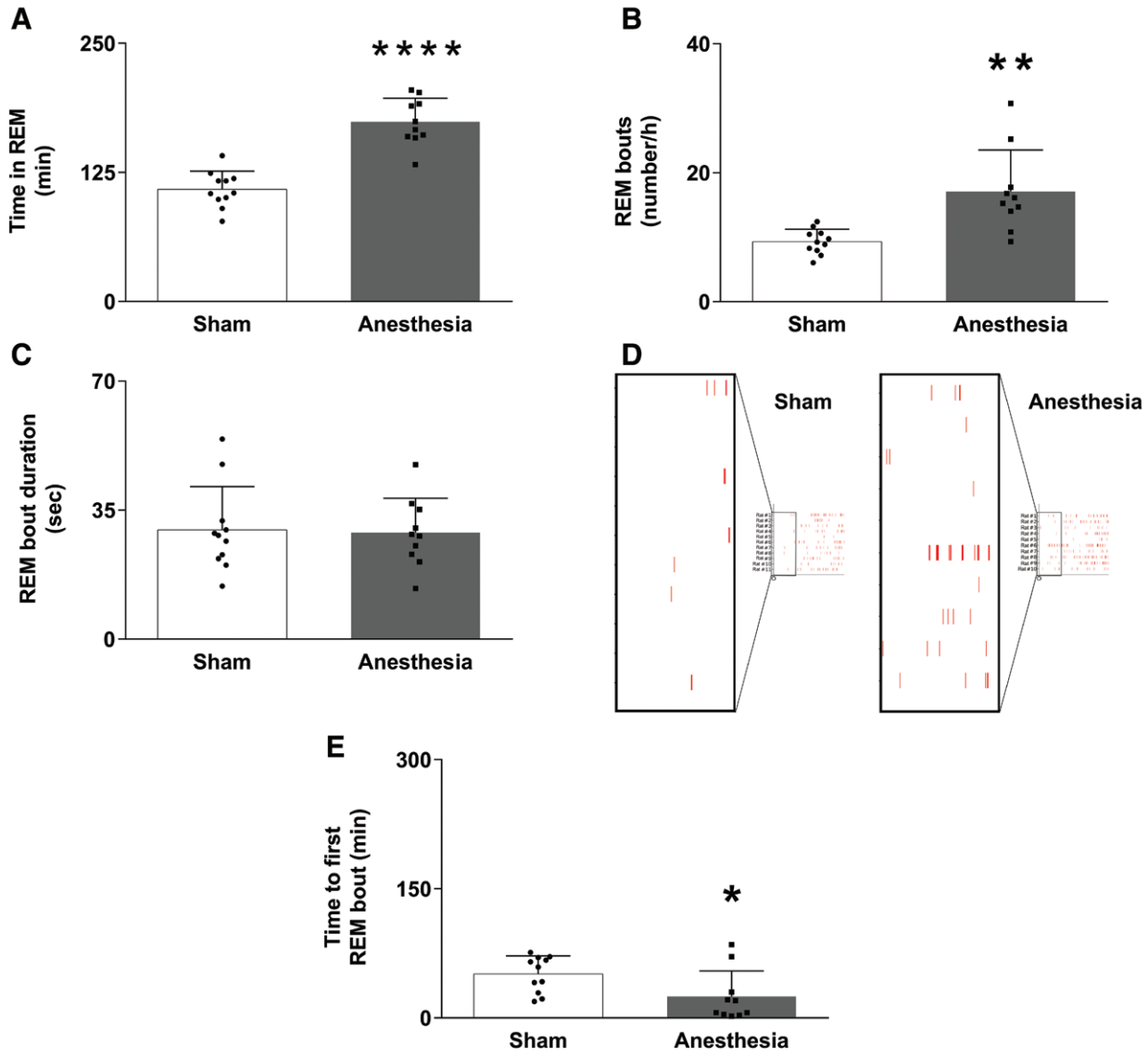

Fig. 7. Effect of neonatal anesthesia on rapid eye movement (REM) sleep. (*A*) The total amount of time spent in rapid eye movement sleep across the 24-h recording period was increased by 40% in Anesthesia 2h rats compared with sham-controls ($P < 0.0001$). (*B*) The number of REM bouts per hour was significantly increased in Anesthesia 2h rats compared with the control condition ($P < 0.001$). (*C*) Neonatal anesthesia did not affect the average duration of REM bouts in Anesthesia 2h animals compared with sham controls ($P = 0.871$). (*D*) Details of the first two h of REM sleep after light onset, from the REM sleep raster plots of sham-control (*left*) and Anesthesia 2h (*right*) rats. Red vertical lines represent REM sleep bouts. Note that although the majority of Anesthesia 2h rats are in REM sleep, only about half of sham-control rats enter the first bout of REM sleep within two h from light onset. (*E*) Anesthesia 2h rats took half the time of sham-controls to enter REM sleep ($P = 0.029$). N = 11 sham-control and 10 Anesthesia 2h rats.

was instead unaffected by the anesthetic treatment (28.9 ± 2.9 s in Anesthesia 2h rats *vs.* 29.7 ± 3.5 in sham-controls, $P = 0.871$, fig. 7C). Figure 7D displays details of the first 2h of rapid eye movement sleep after light onset, taken from the rapid eye movement sleep raster plots of sham-control (left) and Anesthesia 2h (right) rats. As shown in the figure, although the majority of Anesthesia 2h rats were in rapid eye movement sleep within two hours from light onset, only six of 11 sham-control animals had entered the first bout of rapid eye movement sleep. When time from light onset to first rapid eye movement sleep bout was quantified (fig. 7E), we found that Anesthesia 2h rats took half the time of sham-controls to enter rapid eye movement sleep (24.8 ± 9.4 min in Anesthesia 2h rats *vs.* 51.0 ± 6.3 in sham-controls, $P = 0.029$). Of note, rats exposed to a 6h-long anesthesia displayed very similar changes in

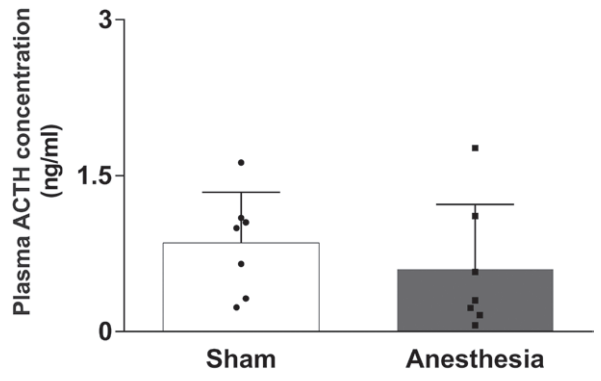

Fig. 8. Electroencephalographic implant surgery and recording do not affect corticotropin concentration in anesthesia-exposed and sham-control rats. Plasma concentration of adrenocorticotropic hormone (ACTH) did not differ between sham-control and Anesthesia 2h rats ($P = 0.414$). N = 7 sham-control and 7 Anesthesia 2h rats.

quantity and structure of rapid eye movement sleep (see the figure, Supplemental Digital Content 5, http://links.lww.com/ALN/B898, representing total amount of time spent in rapid eye movement sleep, number and mean duration of rapid eye movement sleep bouts, and rapid eye movement sleep latency in Anesthesia 6h rats compared to sham-controls). Collectively, these data indicate that (1) the increase in total rapid eye movement sleep time in anesthesia-treated rats is the result of increased consolidation of rapid eye movement sleep bouts and shorter latency to first rapid eye movement sleep bout and (2) even brief anesthetic exposures can evoke substantial electroencephalographic and sleep–wake behavior changes.

Electroencephalographic Implant Surgery and Recording Did Not Affect Corticotropin Concentration in Anesthesia-exposed and Sham-control Rats

Some investigations suggest that activation of the hypothalamus-pituitary-adrenal gland axis in response to biologic stress can interfere with electroencephalographic sleep patterns.[33–36] Therefore, to rule out the possibility that stress may have been responsible for the sleep abnormalities we observed, we measured plasma adrenocorticotropic hormone concentrations in sham-control and Anesthesia 2h rats. Blood was collected between 10:00 AM and 12:00 PM on postnatal days 33 to 35 (the last day of electroencephalographic recording). As shown in figure 8, we found no significant differences in adrenocorticotropic hormone plasma concentration in Anesthesia 2h rats compared with sham-controls (0.6 ± 0.2 ng/ml in Anesthesia 2h rats *vs.* 0.8 ± 0.2 in sham-controls, $P = 0.414$). Likewise, there were no differences in adrenocorticotropic hormone plasma concentrations between Anesthesia 6h and sham-control rats (see the figure, Supplemental Digital Content 6, http://links.lww.com/ALN/B899, representing adrenocorticotropic hormone plasma concentration in Anesthesia 6h rats compared with sham-controls). Therefore, we conclude that the sleep abnormalities we observed after exposure to anesthesia are likely attributable to the effects of the anesthetic drugs *per se*, rather than stress, on endogenous sleep pathways.

Discussion

Our findings indicate that early life anesthesia causes a significant increase in rapid eye movement sleep and disruption of rapid eye movement sleep structure that are present in adolescent rats nearly three weeks after the initial anesthetic exposure, while leaving non–rapid eye movement sleep, wakefulness, and circadian rhythm substantially unchanged.

Several lines of evidence support the notion that anesthetics may interfere with endogenous sleep.[19,20,29–31,37,38] Pick *et al.*[20] reported that adult mice exposed to 6h of sevoflurane or isoflurane exhibited increased rapid eye movement sleep and shortened latency to enter rapid eye movement sleep in the first 18h after anesthesia, but no changes in the amount of non–rapid eye movement sleep or wakefulness. Similarly, sleep-deprived adult rats exposed to 6h of sevoflurane presented a significant increase in rapid eye movement sleep during the first 12h post anesthesia.[30] Relatedly, selectively rapid eye movement sleep-deprived adult rodents subjected to 4h of isoflurane displayed increased rapid eye movement sleep in the 4h postisoflurane.[31] Because a well-known manifestation of sleep deprivation is the ensuing rebound when animals are presented with the opportunity to recover sleep,[39] the rapid eye movement sleep increase observed in these studies was interpreted as a "rebound" (*i.e.*, a homeostatic response to a rapid eye movement sleep deficit that accumulated during anesthetic exposure). Our anesthesia protocols were delivered to infant rats and caused a significant increase in rapid eye movement sleep that was present during adolescence. Although it would be tempting to label such increase in rapid eye movement sleep as a rebound in response to rapid eye movement sleep deprivation accrued during anesthesia, a rebound is by definition limited in duration and should not be present several days after the anesthetic exposure. In other words, even if a rapid eye movement sleep deficit accumulated during anesthesia at postnatal day 7, it would be expected to have dissipated three weeks after the initial exposure.

These considerations call for a different interpretation of our data. Importantly, rapid eye movement sleep is present at high levels at birth in the rat (and humans), begins to decline steeply during the second week of age, and attains adult levels at about the end of the fourth week.[28] In light of this, a possible explanation of our findings is that the changes in rapid eye movement sleep we observe may reflect a delay in the maturation of the neural circuits that regulate sleep induced by anesthetic drugs, such that the normal age at

which adult rapid eye movement sleep levels are attained becomes postponed (*i.e.*, the length of the period during which rapid eye movement sleep remains physiologically elevated is extended). Alternatively, anesthetic drugs administered during a phase of intense brain plasticity may permanently affect the assembly and function of the neural systems that regulate rapid eye movement sleep. In other words, it is possible that early life anesthesia may cause rewiring of the rapid eye movement sleep homeostat and that the rapid eye movement sleep abnormalities we observe may reflect a controller that has recovered to a new set point. Given the crucial role of rapid eye movement sleep for the normal development and function of synapses during brain growth and learning,[21,22,28] either scenario, whether a transient delay in rapid eye movement sleep maturation or a permanent rewiring of rapid eye movement sleep homeostatic regulation circuitry, could have profound implications for proper brain maturation and cognitive development. Clearly, more studies are needed to further elucidate the mechanisms underlying the sleep changes we observe after early life anesthesia.

Our findings indicate a selective effect of anesthetics on quantity and structure of rapid eye movement sleep, as non–rapid eye movement and wake quantity and structure were substantially unaffected by anesthetic exposure. In fact, the increase in rapid eye movement sleep time we observed in our animals led to a decrease that was equally distributed between non–rapid eye movement sleep and wakefulness time. As a result, there were no significant differences in the total time spent in non–rapid eye movement sleep or wakefulness between anesthesia-exposed and sham-control rats. In this respect, our results are consistent with previous studies in which state-specific effects of anesthesia are documented.[19,20,30,31] This diversity of interaction between general anesthetics and sleep–wake states suggests that the brain structures that regulate rapid eye movement sleep, non–rapid eye movement sleep and arousal may be distinct and use dedicated neural circuits.

The suprachiasmatic nucleus serves as the brain's circadian clock. It drives a rhythm that alternates daily between rest and activity cycles and is synchronous with external light cues.[33,34] Our data suggest that early life anesthesia had no influence on the brain's circadian pacemaker. In fact, percent of sleep was appropriately higher during the light cycle (the rest phase) and lower during the dark cycle (the active phase). Conversely, rats spent less time awake during the light cycle and were awake longer during dark hours. These patterns of sleep and wakefulness in anesthesia-treated and sham-control rats are identical to those described in the literature for wild-type animals, suggesting proper timing of rest and activity with external light cues, and therefore normal function of the circadian pacemaker.

One consideration with our experimental design is that rats may have experienced increased stress, as a result of anesthesia exposure or implant surgery, leading to sleep alterations. To minimize this concern, a minimum of 9 days of postsurgical recovery was allowed between electroencephalographic probe placement and electroencephalographic measurement (range 9 to 14 days) in Anesthesia 2h rats. In addition, to rule out an effect of any stress on sleep, we collected blood from electroencephalographic-implanted animals and measured plasma concentration of adrenocorticotropic hormone. We found no differences in adrenocorticotropic hormone between sham-control and anesthesia-exposed animals, ruling out the possibility that stress may have been responsible for the sleep abnormalities we observed. Further, our physiologic monitoring data indicate that RR and HR were significantly different from baseline at the conclusion of the 6 h exposure (RR: 0 h *vs.* 6 h $P = 0.021$; HR 0 h *vs.* 6 h $P = 0.016$). Although it cannot be formally excluded that disturbances in RR or HR are responsible for the changes in rapid eye movement sleep in the Anesthesia 6h group, the observation that anesthesia in the presence of physiologic homeostasis reproduced identical rapid eye movement sleep changes in the Anesthesia 2h group argues against this possibility. Rather, it supports the notion that anesthetic drugs *per se*, and not physiologic derangements, are responsible for the sleep abnormalities we observed.

In this study we examined the effects of a brief (2 h) exposure of infant rats to subanesthetic doses of midazolam, nitrous oxide, and isoflurane on subsequent sleep–wake behavior (Anesthesia 2h group). We also assessed the effects of a longer (6 h) exposure to the same anesthetics (Anesthesia 6h group), in keeping with a substantial body of preclinical work that adopted the same duration of exposure to characterize anesthesia-related morpho-functional changes in the rodent central nervous system.[2–6] Our depth of anesthesia monitoring data indicate that Anesthesia 2h rats remained sedated throughout the exposure, whereas Anesthesia 6h rats were sedated for the first 4 h and achieved a surgical plane of anesthesia during the last 2 h of exposure. Of relevance here, nitrous oxide,[40,41] midazolam,[42,43] and halogenated gases[44] (isoflurane and sevoflurane) at subanesthetic doses are often used to provide sedation to pediatric patients for minor procedures (*i.e.*, dental treatment, superficial wound repair, bone marrow biopsy and diagnostic image acquisition). Building on these considerations, notwithstanding the challenges of translating time and behavior from rodents to humans, our Anesthesia 2h protocol can be taken to model the clinical scenario of procedural sedation, and the Anesthesia 6h protocol of prolonged postoperative sedation.[45,46] Regardless of time equivalences between rodents and humans, our data indicate that even a brief anesthetic exposure in rodents is sufficient to evoke substantial electroencephalographic and sleep–wake behavior changes.

Study Limitations

There are limitations in our study. Because our experimental protocol consisted of a combination of anesthetics,

we cannot draw conclusions on the contribution of each anesthetic agent to the sleep abnormalities we describe. However, the aim of this study was to test the novel hypothesis that administration of anesthesia during a critical period of brain plasticity may disrupt sleep, rather than to focus on the specific effects of each anesthetic agent on sleep. Further studies are needed to characterize the impact of each component of our anesthetic protocol on sleep–wake behavior. Additionally, although rodent models have significantly advanced the field of sleep research, our data regarding sleep–wake states in rodents will warrant verification in humans.

Conclusions and Scientific Implications

We demonstrate that a brief exposure to a subanesthetic combination of midazolam, nitrous oxide and isoflurane during a critical period of brain plasticity results in rapid eye movement sleep increase and perturbation of rapid eye movement sleep architecture that are present in adolescent rats several days after the anesthetic encounter. These results are significant for many reasons. First, given the association between rapid eye movement sleep, synaptic plasticity and memory consolidation, rapid eye movement sleep disruption could represent an unexplored mechanism contributing to the learning disabilities observed after neonatal anesthesia. Second, it may represent a novel marker of anesthetic effects on the developing brain and should be evaluated as relevant outcome in future studies of anesthetic-induced neurotoxicity. Third, the study of the electroencephalographic changes during and after anesthesia may provide insight into the mechanisms by which anesthetics modulate neuronal function.

Acknowledgments

The authors thank Eric M. Davis, M.D. (Department of Internal Medicine, Division of Pulmonary and Critical Care Medicine, University of Virginia Health System, Charlottesville, Virginia) for his assistance with accuracy validation of electroencephalographic scoring in rats; Mark E. Smolkin, M.S. (Department of Public Health Sciences, University of Virginia, Charlottesville, Virginia) for his assistance with statistical analysis of sleep-wake data; Douglas A. Bayliss, Ph.D. (Department of Pharmacology, University of Virginia, Charlottesville, Virginia) for his critical review of the manuscript; and Bianca Ferrarese, M.D. (Department of Anesthesiology, University of Virginia, Charlottesville, Virginia and Department of Anesthesiology, University of Padova, Padova, Italy) for her help with assessment of anesthesia depth and physiologic monitoring during anesthesia.

Research Support

Supported by grant Nos. NIGMS-K08GM123321-01 (to Dr. Lunardi) and R01 HD089999 (to Dr. Zuo) from the National Institutes of Health (Bethesda, Maryland); grant No. GF13062 from the International Anesthesia Research Society (San Francisco, California; to Dr. Lunardi); and research funds from the Department of Anesthesiology, University of Virginia, Charlottesville, Virginia.

Competing Interests

The authors declare no competing interests.

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