Minireview

Neonatal exposure to a neuroactive steroid alters low-frequency oscillations in the subiculum

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Impact statement

Long-term functional consequences of neonatal exposure to general anesthetics at the level of intact neuronal networks are not well studied. Here, we explored the lasting effects of a novel anesthetic and neuroactive steroid 3β-OH on sleep architecture, as well as neuronal oscillations and synaptic plasticity in intact rat subiculum, a part of hippocampal formation that is heavily involved in cognitive functions. We report that neonatal exposure to 3β-OH decreased subicular delta and sigma oscillations during non-rapid eye movement sleep without altering sleep architecture and subicular synaptic plasticity. In contrast, our previous study found that neonatal exposure to a common anesthetic agent ketamine increased subicular gamma oscillations during non-rapid eye movement sleep and profoundly suppressed subicular long-term potentiation in adolescent rats. We posit that exposure to different sedative/hypnotic agents during a critical period of brain development may induce distinct functional changes in subiculum circuitry that may persist into adolescent age.

Abstract

Preclinical studies have established that neonatal exposure to contemporary sedative/hypnotic drugs causes neurotoxicity in the developing rodent and primate brains. Our group recently reported that novel neuroactive steroid (3β,5β,17β)-3-hydroxyandrostane-17-carbonitrile (3β-OH) induced effective hypnosis in both neonatal and adult rodents but did not cause significant neurotoxicity in vulnerable brain regions such as subiculum, an output region of hippocampal formation particularly sensitive to commonly used sedatives/hypnotics. Despite significant emphasis on patho-morphological changes, little is known about long-term effects on subicular neurophysiology after neonatal exposure to neuroactive steroids. Hence, we explored the lasting effects of neonatal exposure to 3β-OH on sleep macrostructure as well as subicular neuronal oscillations *in vivo* and synaptic plasticity *ex vivo* in adolescent rats. At postnatal day 7, we exposed rat pups to either 10 mg/kg of 3β-OH over a period of 12 h or to volume-matched cyclodextrin vehicle. At weaning age, a cohort of rats was implanted with a cortical electroencephalogram (EEG) and subicular depth electrodes. At postnatal day 30–33, we performed *in vivo* assessment of sleep macrostructure (divided into wake, non-rapid eye movement, and rapid eye movement sleep) and power spectra in cortex and subiculum. In a second cohort of 3β-OH exposed animals, we conducted *ex vivo* studies of long-term potentiation (LTP) in adolescent rats. Overall, we found that neonatal exposure to 3β-OH decreased subicular delta and sigma oscillations during non-rapid eye movement sleep without altering sleep macrostructure. Furthermore, we observed no significant changes in subicular synaptic plasticity. Interestingly, our previous study found that neonatal exposure to ketamine increased subicular gamma oscillations during non-rapid eye movement sleep and profoundly suppressed subicular LTP in adolescent rats. Together these results suggest that exposure to different sedative/hypnotic agents during a critical period of brain development may induce distinct functional changes in subiculum circuitry that may persist into adolescent age.

Keywords: 3β-OH, hypnosis, brain development, EEG, long-term potentiation

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Introduction

Each year, millions of infants and young children are exposed to sedative/hypnotic and anesthetic agents in the operating rooms and intensive care units.[1] Based on their mechanism of action, these agents are either positive allosteric modulators (PAMs) of neuronal γ-aminobutyric acid subtype A ($GABA_A$) receptors, such as propofol, or *N*-methyl-D-aspartate (NMDA) receptor antagonists, such as ketamine and nitrous oxide.[2,3] Several studies have demonstrated that exposure to both classes of sedative/hypnotic and anesthetic drugs early in life is associated with profound neurotoxicity and consequently cognitive and socio-affective impairments in rodents and non-human primates.[4–8] The lasting effects of sedative/hypnotic and anesthetic drugs in infants and young children is still under intense investigation. Similar to studies conducted in non-human primates,[9] some human retrospective studies suggest that a single, brief exposure may not result in neurodevelopmental delay.[10–12] However, it has been suggested that repeated exposures to anesthetics in early childhood may lead to cognitive and importantly, socio-affective impairments as detected later in life.[10,13,14] Prolonged exposures of infants to sedative/hypnotic agents are more common in the setting of intensive care units then in the operating rooms, but they remain poorly studied in clinical research.[15] Hence, further preclinical research is warranted to elucidate cellular mechanisms of long-lasting effects of currently available sedative/hypnotic agents on neuronal function and to inform possible therapeutic strategies that could be used to make clinical anesthesia safer for our youngest patient population.

We recently proposed that developmental cognitive dysfunction after neonatal exposure to sedatives/hypnotics can be ameliorated or completely avoided using neuroactive steroids. One such agent is (3β,5β,17β)-3-hydroxyandrostane-17-carbonitrile (3β-OH). Unlike many other sedatives/hypnotics, 3β-OH inhibits neuronal voltage-gated T-type calcium currents but lacks any direct PAM properties on $GABA_A$ receptors or inhibitory effect on NMDA receptors.[7,16] Importantly, when we compared safety of lengthy exposure to 3β-OH *vis-à-vis* ketamine in neonatal rats, we found that 3β-OH did not induce neuroapoptosis in the developing brain and did not cause learning/memory impairments later in life.[7] In regard to other neuroactive steroids, we also demonstrated that alphaxalone (3α-hydroxy-5α-pregnane-11,20-dione), although a canonical PAM of $GABA_A$ receptors, also did not induce developmental neuroapoptosis in rat pups, presumably due to presynaptic inhibition of GABA release.[17] Importantly, alphaxalone has been used in veterinary medicine in many countries including the United States[18] and is undergoing clinical trials under trade name Phaxan marketed by Drawbridge Pharmaceuticals (Malvern, VIC, Australia).[19] The ongoing use of alphaxalone continues to encourage future development and further studies of possible long-term effects of synthetic neuroactive steroids with hypnotic/anesthetic properties.

Prevailing literature supports the idea that common general anesthetics cause neurodegeneration and possibly other long-term changes in the developing mammalian brain, especially in the subiculum. However, until recently, there has been little research on how exposure to neonatal sedative/hypnotics and anesthetics affects long-term sleep macrostructure, corresponding neuronal oscillations, and synaptic plasticity in hippocampal circuitry. This is particularly relevant since it is well established that neuronal T-type channels play an important role in rhythmic neuronal oscillations and maintain sleep architecture.[20] Furthermore, our previous studies have shown that 3β-OH inhibits T-currents in the thalamic and subicular neurons in acute brain slices from neonatal rats.[7,21] Together these studies may help to further our understanding of underlying mechanisms by which exposure to different classes of sedative/hypnotics and anesthetics may contribute to poor neurocognitive outcomes. Hence, we designed this study to examine the long-term effects of neonatal exposure to 3β-OH on sleep architecture and synaptic plasticity in the rat subiculum. We chose to focus on subiculum for two main reasons: (1) our previous studies have shown that the subiculum is exquisitely vulnerable to neurotoxic insult from neonatal anesthesia with ketamine[7] and (2) subiculum is a key relay center between the hippocampal complex and various cortical and subcortical structures and is heavily implicated in memory processing.[22,23] Because 3β-OH, unlike ketamine, does not cause developmental neurotoxicity in the neonatal subiculum at equipotent doses,[7] we hypothesized that exposure to 3β-OH during the peak of synaptogenesis (at postnatal day 7 in rats) may not disrupt critical neuronal oscillations in the subiculum *in vivo* and synaptic plasticity *ex vivo*. To examine our hypothesis, we used *in vivo* cortical electroencephalogram (EEG) recordings and subicular local field potential (LFP) recordings to characterizing sleep architecture (macrostructure) and neuronal oscillations during sleep. In addition, we used *ex vivo* extracellular recordings in acute hippocampal slices to measure changes in long-term potentiation (LTP) as a surrogate for memory formation in the subiculum.

Materials and methods

Animals

Sprague-Dawley rat pups of both sexes were obtained at age postnatal day 5 (Envigo, Indianapolis, IN, USA) and housed with their mothers until experiment initiation. Animals were housed on a 14:10 light–dark cycle and given access to food and water *ad libitum.* All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Colorado Anschutz Medical Campus and adhered to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Drugs

The 3β-OH was synthesized as previously described[7] and dissolved in 15% (2-hydroxypropyl)-β-cyclodextrin solution (cyclodextrin) (Santa Cruz Biotechnology Inc., Dallas, TX, USA). This mixture was delivered via intraperitoneal (i.p.) injection at a 10 mg/kg dosage and at a volume no greater than 2 μL/gram body weight.

Exposures

At postnatal day 7 (P7), rat pups were removed from their home cages and randomly assigned to receive either 10 mg/kg 3β-OH or 15% cyclodextrin sham i.p. injections

(Figure 1(A), P7 timepoint). The P7 timeframe aligns with the peak synaptogenesis of animals when they are most vulnerable to effects of anesthesia.[4,24] Animals were injected every 2 h for 12 h (6 total injections) at 10 mg/kg. During these injections, animals were placed on a heating pad set to 35°C to keep the pups warm. We did not assess physiological parameters of animals during injections. However, throughout the injection timeline, the pups showed no signs of hypoxia physically; the pups appeared pink and healthy not gray or pallid. The dose of 10 mg/kg was chosen based on previous work from our group showing that the effects of 10 mg/kg of 3β-OH is equipotent with the effects of 40 mg/kg of ketamine, a common anesthetic and a drug we have used in our previous sleep study.[24] Importantly, we showed that at this dose and administration regimen 3β-OH did not induce significant neuroapoptosis in P7 rat pups.[7] After the 12-h exposure, the pups were allowed to recover and were then returned to their home cages with their mothers.

It is also important to mention that 3β-OH at the dose 10 mg/kg i.p. had a sedative/hypnotic effect and not full anesthetic effect in the rat pups. Although all rat pups were not showing visible signs of spontaneous movement and righting reflex, we observed that they still retained the subanesthetic withdrawal reflex upon pinching their leg with a forceps (data not shown). Thus, our data show long-term neurophysiological effects of subanesthetic doses of 3β-OH comparable to subanesthetic equipotent dose of ketamine at 40 mg/kg i.p. as described in Manzella *et al*.[24] Hence, our experimental paradigm is more relevant to the sedative/hypnotic doses used for prolonged sedation in the pediatric intensive care unit setting, rather than operating rooms.

Electrode implantation

To study the effects of an early sedative/hypnotic exposure in a neonatal model on sleep architecture, a subset of both 3β-OH animals and sham animals were implanted at wean age (P21–23, Figure 1(A)) via stereotaxic surgery. Initial anesthesia was induced by 3% isoflurane via inhalation and maintained with 1–2% isoflurane. Since all animals were past the critical point for anesthesia induced neurotoxicity, briefly putting the sham animals under isoflurane for surgery was not concerning. A screw electrode was implanted at stereotaxic co-ordinates AP: −2.8, ML 3.0 in the barrel cortex range. LFPs in the dorsal subiculum were obtained by a depth electrode implantation at co-ordinates AP: −4.8, ML 2.4, DV 2.5. Ground and reference screws were implanted caudal to lambda: just left of the midline was the ground electrode and just to the right was the reference electrode. To measure electromyography (EMG), an insulated silver wire hook was inserted into the nuchal muscle. Dental acrylic gel was used to fix the electrodes to the skull, forming the overall EEG headpiece. Banamine (2.5 mg/mL subcutaneously) was given to animals postoperatively for analgesia every 24 h for 48 h.

Postsurgery, animals were housed individually and given a week to recover and adjust to the headpieces. EEG acquisition and recording took place between P30 and P33 (P30–33 timeframe, Figure 1(A)). Pinnacle system (Pinnacle Technology, Inc., Lawrence, KS, USA) was used for EEG signal recording along with synchronized time-locked videos. EEG signals were acquired from 1 to 500 Hz using a band-pass filter, digitized at 2000 Hz and stored on a hard drive for offline analysis.

Sleep architecture and analysis

Analysis for EEG data was taken from day 3 of recording (age of P32) so that the animals had ample time to acclimate to the recording device. Recordings were separated into light and dark cycles, and 3 continuous hours from each cycle was used for analysis. Each cycle was analyzed separately first and then combined together for a full analysis. Number of animals in each group for these studies was as follows: experimental group $N = 8$ (3 females and 5 males) and vehicle (cyclodextrin) group ($N = 11$, 7 females and 4 males) rats.

All sleep scoring was done manually in Sirenia Sleep Pro (Pinnacle Technology, Inc.) in 10-s epochs. The manual scoring was aided by epoch-to-epoch visualization of spectral plots along with time-locked videos. The different sleep stages were divided into wake, non-rapid eye movement (NREM), and rapid eye movement (REM) sleep. Along with these separate divisions, we also combined all NREM and REM sleep together for total sleep (sleep) to compare sleep to wake behavior. Wake was scored by the presence of mixed amplitude, high-frequency oscillations and significant movement in the EMG (Figure 1(B)). Due to how large the variability for neuronal oscillations is for NREM, this was further divided into N1 and N2 sleep. N1 was categorized by the presence of high-amplitude, low-frequency oscillations in the EEG and minimal signal in the EMG (Figure 1(C)). N2 was differentiated from N1 by a substantial amount (at least 50% of the epoch) containing delta (1–4 Hz) oscillations.[24–26] This was confirmed by the slow (<1 Hz) and delta (1–4 Hz) power being dominant in the epoch's spectral plot. Finally, REM sleep was categorized by having a very similar visual appearance (high frequency) to that of wake in the cortical EEG, but the EMG signal is flatlined, and when the spectral plot is looked at theta waves (4–8 Hz) are the prominent waves found (Figure 1(D)). Experimenter was blinded to treatment condition of all animals analyzed; only animals with postmortem confirmation of electrode placement were used in this study.

As in Manzella *et al*.,[24] we analyzed sleep architecture using a number of behavioral outcomes: number of episodes, length of each episode (in seconds), stage length (in minutes), and transitions from one stage to another. A single sleep episode is defined as one period of time that an animal spent in one sleep stage before transitioning to another stage (e.g. wake to NREM). We measured the number of episodes separately from the episode length because it can appear that there is no overall change between time spent in one stage versus another, but this can mask sleep fragmentation. By sleep fragmentation, an example is that one group has more episodes of N1 but a shorter episode duration. By separating these out, we are able to look not only at the stages of sleep themselves, but also at how (and if) the sleep is fragmented. Episode length was calculated by summing the total time spent in each episode and dividing that by the number of episodes. Mean stage length was compared between each group; each time there was a switch from one stage to another, and it was counted as a transition.

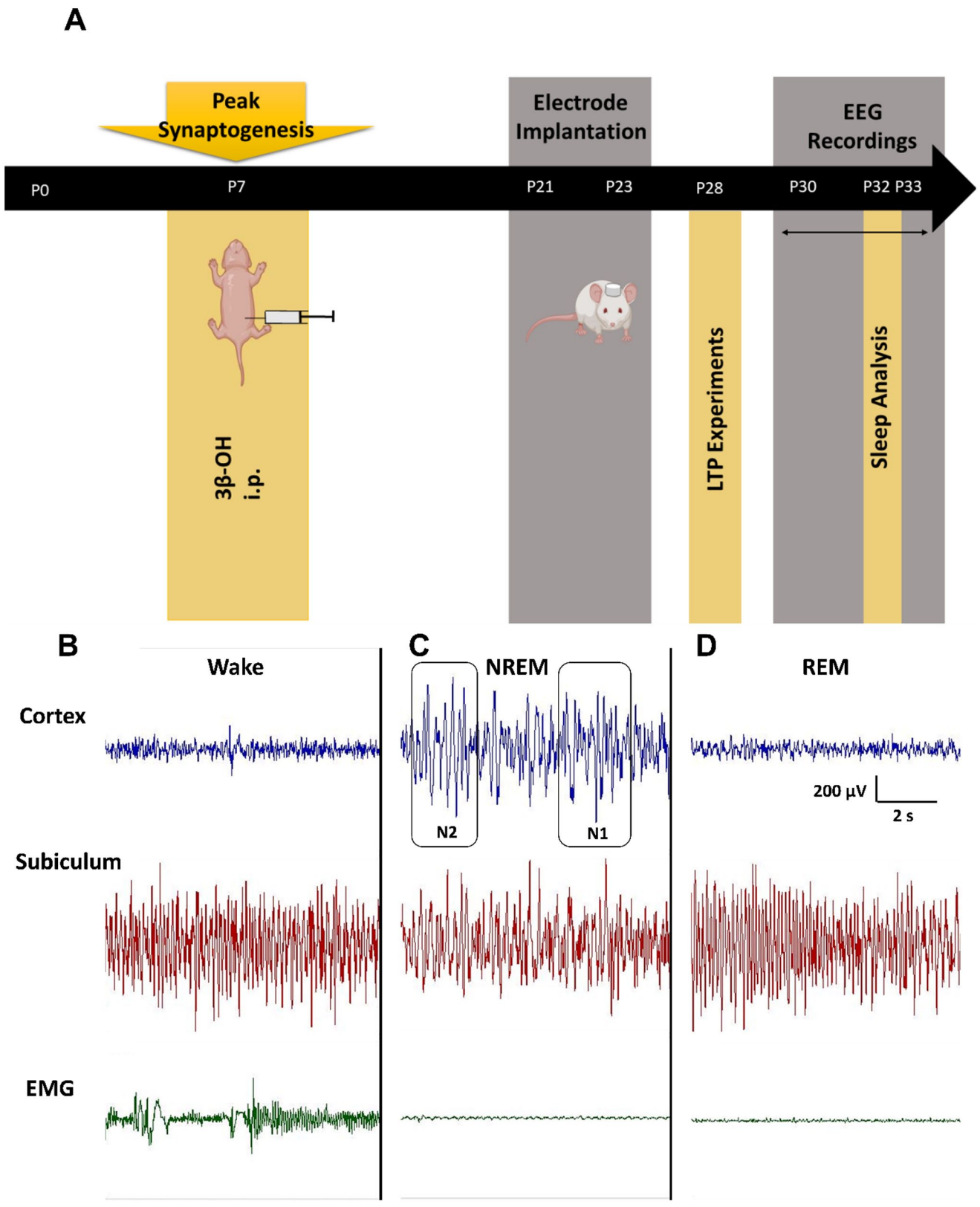

Figure 1. (A) Schematic timeline representation of events for the experiment. Exposure to the 12-h regimen of 3β-OH at 10 mg/kg occurred at peak synaptogenesis of P7. A small subset of animals was used for LTP experiments at P28, the rest were implanted between P21 and P23 after weaning from their mothers. Recording was done from P30 until P33, analysis was taken from P32. (B–D) Representative EEG traces of subicular and cortical EEG and of EMG signals that were used in analysis along with time-locked videos. (B) Traces during wake; the animal was freely moving and is indicated by high-frequency low-amplitude subicular and cortical traces and high-frequency EMG. (C) Representative traces of NREM sleep shown through low-frequency high-amplitude cortical traces with little activity in the EMG. The inset highlights the difference between N1 and N2, in that N2 contains a higher amount of delta oscillations (>50% of the epoch containing delta). (D) REM representative trace shown to have a high-frequency cortical EEG very similar to those found in wake but with a flat line in the EMG.

Power analysis

Power spectra values were obtained using the fast Fourier transform (using Sirenia Sleep Pro software) with the EEG cortex waveform data and LFP waveform data from the subiculum. The data were divided into seven frequency bands: delta (0.5–4 Hz), theta (4–8 Hz), alpha (8–12 Hz), sigma (9–15 Hz), beta (12–30 Hz), low gamma (30–59 Hz), and high gamma (61–100 Hz). Absolute power was summed for the cycle as a whole, and each power spectra value was divided per the number of episodes for the particular sleep stage.

LTP studies

The methods used in this study to record LTP from subiculum are identical to those described in Manzella *et al.*[24] However, we include details here for the sake of completeness of this article. Hippocampal slices were prepared from rats at age of P28 after neonatal exposure to 3β-OH (10 mg/kg) or vehicle treatment at age of P7. Rats were anesthetized with 3% isoflurane in an O_2-enriched chamber. Rats were transcardially perfused with ice-cold (2–5°C) oxygenated (95% O_2/5% CO_2) artificial cerebral spinal fluid (ACSF) for 2 min prior to decapitation. The composition of ACSF was the following (in mmol/L): 126 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.3 NaH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgCl_2$, and 12 glucose. Horizontal hippocampal slices (300 µm thick) were cut with a Vibratome 1200S (Leica, Wetzlar, Germany) and transferred to a holding chamber containing ACSF for at least 1 h before recording.

Synaptically evoked field potentials were recorded from hippocampal slices that were placed on a temperature controlled (31 ± 0.5°C) interface chamber perfused with ACSF at a rate of 1.5 mL/min. For subiculum extracellular recordings, a bipolar electrode was placed in stratum oriens at the very end of the CA1 region. Field excitatory postsynaptic potentials (fEPSPs) were produced by recording in the stratum-radiatum of the distal dendrites of subicular neurons. The fEPSPs were adjusted to 50% of the maximum slope, and test pulses were evoked every 20 s. A 20-min stable baseline was established before delivering a theta-burst stimulation (TBS) train of four pulses delivered at 100 Hz in 30 ms bursts repeated 10 times with 200 ms interburst intervals. Following TBS, the fEPSP was recorded for 60 min. The averaged 10 min slope from 50 to 60 min after TBS was divided by the average of the 10-min baseline (set to 100%) prior to TBS to determine the amount of potentiation. Analog fEPSPs were amplified (1000×) and filtered through a preamplifier (Model LP511 AC, Grass Instruments, West Warwick, RI, USA) at 1.0 kHz, digitized at 10 kHz and stored on computer for later off-line analysis (Clampfit 10.4, Axon Instruments, Forest City, CA, USA). The derivative (dV/dT) of the initial fEPSP slope was measured. For time course graphs, normalized fEPSP slope values were averaged and plotted as the percent change from baseline. Two electrophysiologists (RMD and JEO) were blinded to the treatment group (vehicle vs experimental) and independently verified all LTP results in this report. Sixteen rats were used in total in this study. We recorded with no more than two experiments per animal.

Data and statistical analysis

Two-tailed independent samples *t*-tests were used to compare groups when equal variances were assumed; unpaired *t*-test with Welch's correction were used for groups when equal variances were not assumed (*F*-test for variance, $P < 0.05$), and GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA) was used for all analyses.

Results

The summary graphs on Figure 2 show the effects of neonatal exposure to 3β-OH on different sleep macrostructure parameters by comparing the experimental (3β-OH treated) group ($N = 8$, 3 females and 5 males; blue symbols) and vehicle (cyclodextrin) treated ($N = 11$, 7 females and 4 males; maroon symbols) rats. As depicted in Figure 2, neonatal exposure to 3β-OH did not result in any significant changes in sleep macrostructure across all of the sleep stages. Specifically, there were no changes in the number of episodes spent in each stage or the length of each episode (Figure 2(A) and (B), respectively), and there were also no differences in the total length of each stage (Figure 2(C)). Since these results were consistent across the light and the dark cycle, the data are presented in Figure 2(A) to 2(C) as combined across both cycles. Finally, there were no differences in the number of transitions between stages during the light cycle, dark cycle, or across both combined (Figure 2(D)).

Next, we compared any lasting effects of neonatal exposure to 3β-OH versus vehicle group on LFP delta power spectra in the subiculum of adolescent rats (Figure 3). We found that there was a trend for a decrease in an absolute delta power in 3β-OH exposed (maroon symbols) animals when compared to vehicle-treated pups (blue symbols) cohort in the N1 stage of NREM sleep during individual light (Figure 3(A)) and dark (Figure 3(B)) cycles; this effect reached statistical significance only when these two cycles were combined (Figure 3(C)). Furthermore, Figure 4 summarizes lasting effects of neonatal exposure to 3β-OH (maroon symbols) and vehicle (blue symbols) on absolute sigma power in the subiculum of adolescent rats. We detected about twofold decrease in the sigma power in 3β-OH group when compared to the vehicle (cyclodextrin) in N1 stage of NREM sleep that was trending in the individual light cycle (Figure 4(A)) but was significant in the individual dark cycle (Figure 4(B)) and when both of these cycles were combined (Figure 4(C)).

While we did not observe changes in delta oscillations, we noticed a significant decrease in sigma oscillations during N2 stage of NREM sleep in the simultaneous EEG recordings from the barrel cortex in the same animals (data not shown). In addition, we also independently analyzed changes in gamma, theta, alpha, and beta oscillations in both subiculum and barrel cortex. However, we did not note any statistical differences between 3β-OH-treated rats and vehicle (cyclodextrin) treated rats across those frequency bands (data not shown).

Finally, to determine any lasting effect of 3β-OH on hippocampal synaptic plasticity that may underlie cognitive functions in developing brain, extracellular field recordings

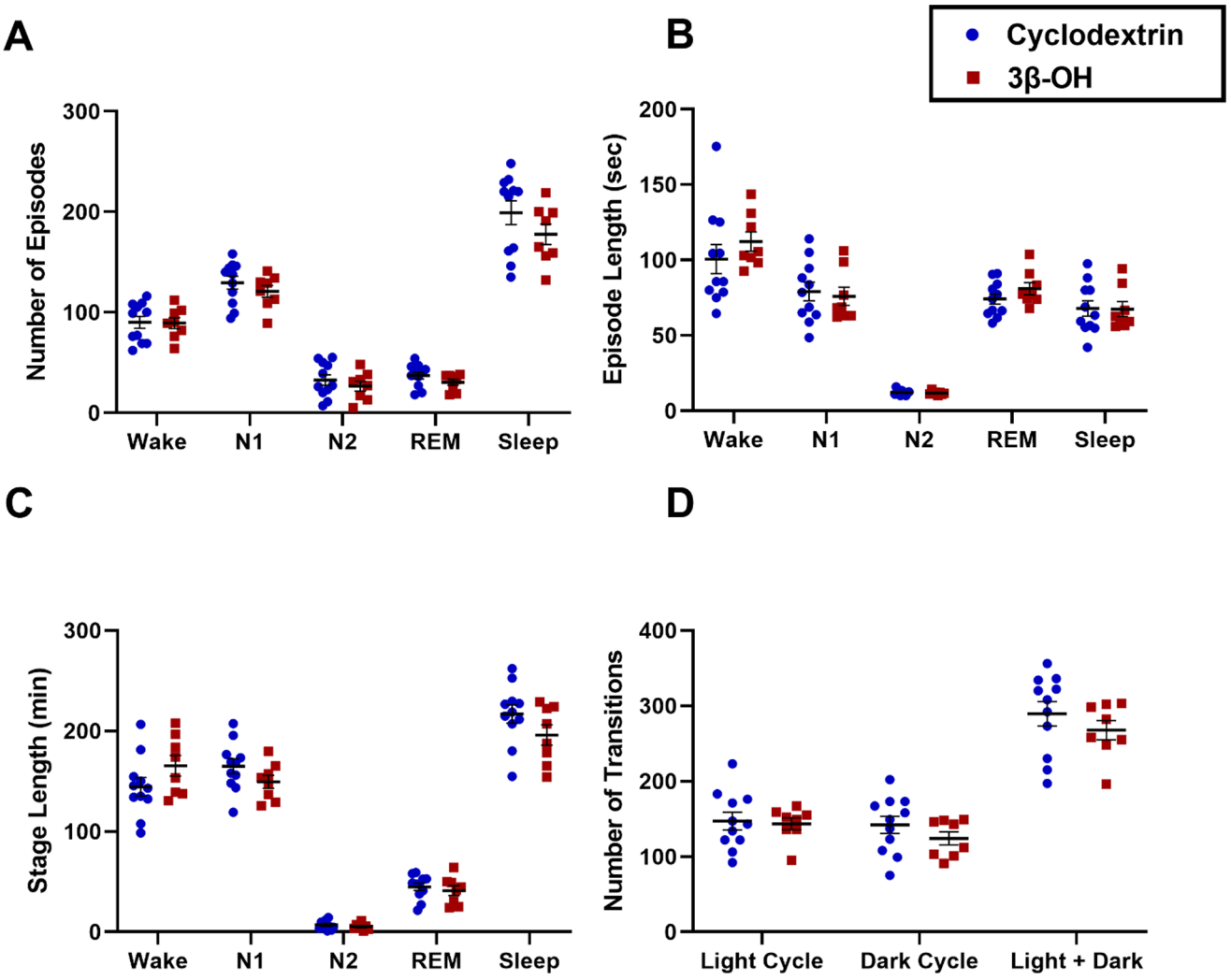

Figure 2. Overall sleep architecture of vehicle and 3β-OH exposed neonates. All graphs represent the combined light and dark cycles of the animals as there were no significant differences found when separated. (A) There were no noted differences in the number of episodes in the wake ($P=0.929$), N1 ($P=0.349$), N2, ($P=0.421$), REM ($P=0.175$), and total sleep stages ($P=0.209$). (B) We also did not note any changes in the episode length (in seconds) between the wake ($P=0.363$), N1 ($P=0.728$), N2, ($P=0.599$), REM ($P=0.216$), and total sleep stages ($P=0.960$). (C) In turn, the length of each stage did not differ significantly from one another either wake ($P=0.151$), N1 ($P=0.148$), N2, ($P=0.376$), REM ($P=0.541$), and total sleep stages ($P=0.145$). (D) We also measured how often the animals would transition from one stage to another, and there were no significant differences found between the light ($P=0.817$), dark ($P=0.256$) cycles, as well as when we combined the light and dark cycles ($P=0.340$).

of LTP from subicular neurons *ex vivo* were analyzed from P28 rats. The data are summarized in Figure 5 where we compared groups following neonatal exposure to 3β-OH (maroon symbols) and vehicle (blue symbols). Figure 5(A) shows a timeline of these experiments where following a stable 20-min baseline, we applied a brief TBS (40 pulse TBS) to evoke LTP response. The results showed that in both groups the amplitude of fEPSP was increased about 2.5-fold when compared to the baseline values with mostly overlapping timepoints up to 80 min of recordings. Figure 5(B) shows a bar graph summary of these results. We conclude that there was no significant impairment of LTP in the experimental group exposed to 3β-OH when compared to the vehicle group (cyclodextrin).

Discussion

The ability of sedative/anesthetic drugs to induce safe and reversible loss of consciousness is of paramount importance. Interestingly, despite their clinical use for nearly two centuries, the mechanisms whereby canonical sedative/hypnotic agents produce loss of consciousness are not well understood. Having said that, it is becoming clear that most common cellular targets are either glutamate (Glu)-mediated excitatory transmission via inhibition of NMDA receptors (e.g. ketamine, nitrous oxide [N_2O]) and/or enhancement of neuronal inhibition mediated via $GABA_A$ receptors (e.g. isoflurane, sevoflurane, midazolam, propofol). Unfortunately, their effects on NMDA and $GABA_A$ receptors are also believed to be the basis for their developmental neurotoxicity. Indeed, both recent preclinical[4–9] and clinical[10,13,14] studies strongly suggest that common sedatives/hypnotics may harm the developing mammalian brain. Since the majority of clinically used sedatives and hypnotics have been shown to induce developmental neurotoxicity, there is an increasing realization that there is a need for the development of safer anesthetic agents. We recently postulated that developmental cognitive dysfunction after neonatal exposure to sedatives/hypnotics can be ameliorated or completely

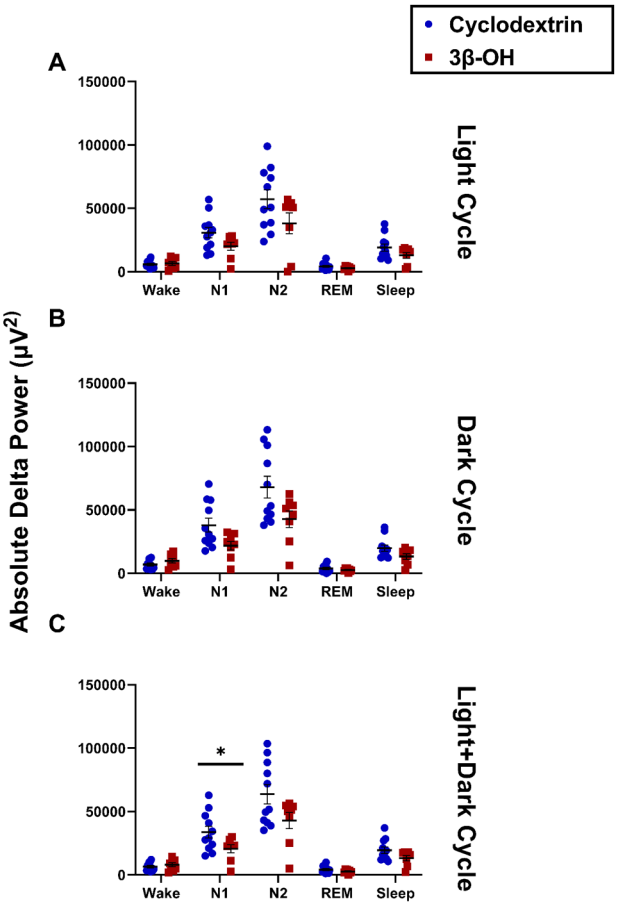

Figure 3. 3β-OH decreases power in delta oscillations in N1 sleep stage in subiculum. (A and B) No significant changes in the delta oscillations' power found between any of the stages in the light or dark cycles when analyzed separately. (C) When the light and dark cycles were combined for analysis, wake, N2, REM, and total sleep stages have no significant differences between the stages ($P=0.379$, $P=0.063$, $P=0.159$, $P=0.087$, respectively), but sleep stage N1 is significantly decreased in animals neonatally treated with 3β-OH ($P=0.047$). *$P<0.05$.

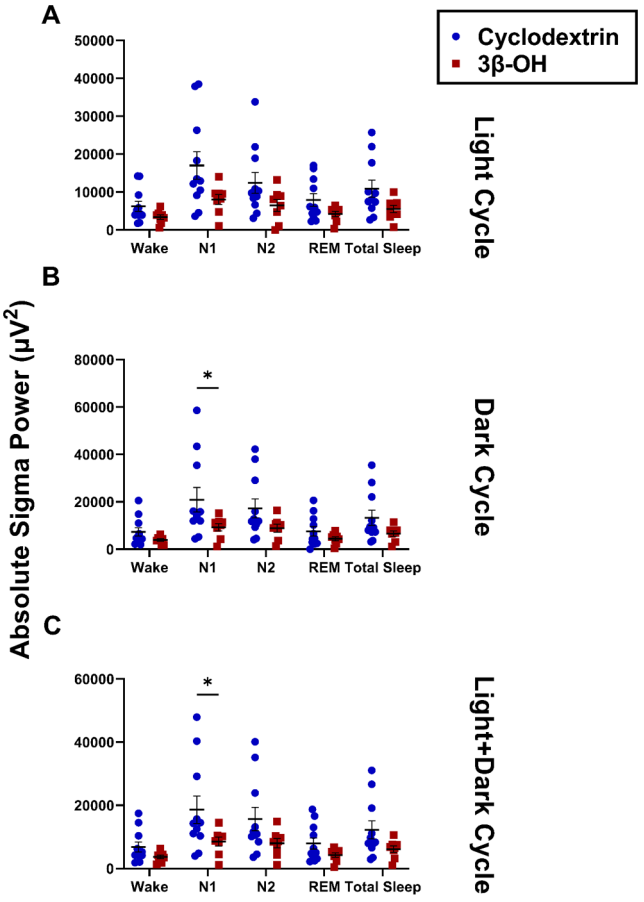

Figure 4. 3β-OH decreases the subicular sigma power during N1. (A) No significant differences were found during the light cycle during wake ($P=0.152$), N1 ($P=0.075$), N2, ($P=0.104$), REM ($P=0.272$), and total sleep stages ($P=0.075$). (B) However, during the dark cycle, we see no significant differences in wake, N2, REM, and total sleep ($P=0.201$, $P=0.091$, $P=0.545$, and $P=0.109$, respectively), but during sleep stage N1, we found a significant decrease in subicular sigma ($P=0.020$). (C) When combining the light and dark cycles total time analyzed, we find a similar pattern found in panel (B) that there are no significant differences in wake, N2, REM, and total sleep ($P=0.177$, $P=0.109$, $P=0.395$, and $P=0.062$, respectively) yet N1 is significantly decreased in subicular power ($P=0.033$). *$P<0.05$.

avoided using neuroactive steroids. Of particular interest for this study is 3β-OH known to inhibit neuronal voltage-gated T-type calcium currents without any direct PAM properties on $GABA_A$ receptors or inhibitory effect on NMDA receptors.[7,16] Importantly, we found that 3β-OH did not induce neuroapoptosis in the developing brain, including subiculum when used in exactly the same protocol as in this study.[7]

Although prevailing literature has focused on the mechanisms of neuroapoptosis, the consequences of maladaptive plasticity of neural networks that survived initial apoptotic insult caused by sedatives/hypnotics are not well studied. Hence, further preclinical research is warranted to elucidate the cellular mechanisms that underlie the long-lasting neurodevelopmental effects of currently available sedative/hypnotic agents and to inform possible therapeutic strategies that could be used to make clinical anesthesia practice safer in the operating rooms and intensive care units. Currently, very little is known about how early life exposures to sedative/hypnotic drugs may affect sleep-wake behaviors and their corresponding neuronal oscillations. This is important since neuronal oscillations that underlie the sleep-wake cycle play a critical role in neuronal development, cognitive processing, socio-emotional adaptivity and homeostatic sleep drive.[27,28] Despite acute insult to key sleep centers during the critical period of brain development in rodents, we did not find any effects of 12-h-long neonatal ketamine exposure on sleep macrostructure.[24] Similarly, we found that a 6-h-long neonatal exposure to neurotoxic concentrations of isoflurane at 1.5% caused minimal changes in cortical beta oscillations but did not result in changes in sleep macrostructure in adolescent rats.[29] This is in agreement with this study that exposure to 3β-OH for 12 h in P7 rat pups did not cause any notable changes in sleep macrostructure in adolescent rats.

Although we did not note any changes in sleep macrostructure with different anesthetic treatments, we did observe changes in neuronal oscillations in the subiculum during NREM sleep. The subiculum receives unidirectional outgoing information from CA1 area and is in an important position to serve as a relay center between the hippocampal complex and numerous cortical and subcortical structures.[22] Low- and high-gamma oscillations generated from various

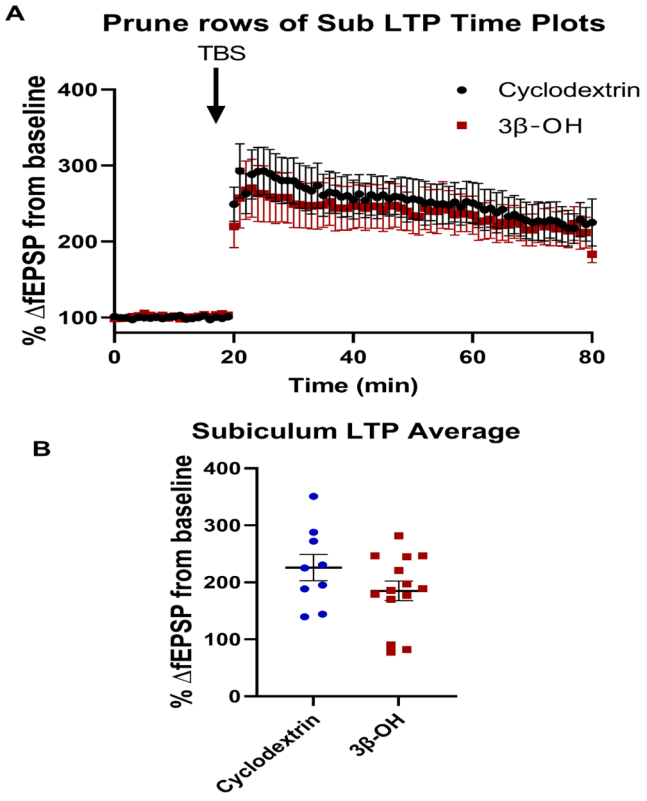

Figure 5. Twelve hours of neonatal exposure to 3β-OH does not impair synaptic plasticity in adolescent rats. (A) Time plots of fEPSP slope from CA1 to subiculum pathway in P28 rats exposed to neonatal cyclodextrin ($n = 9$) or 3β-OH ($n = 12$). TBS arrow indicates the timing of theta-burst stimulation (40 pulses, 100 Hz). (B) Quantification of change in fEPSP slope after 60 min following TBS (dark box) normalized to baseline (light box) in ipsilateral slices and contralateral slices, set at 100%. Rats exposed to neonatal 3β-OH showed no significant impairment in LTP ($P = 0.160$). Each point represents a hippocampal slice (experiment) that was recorded with no more than two experiments per animal used. Sixteen rats were used in total, blinded to the experimenter.

regions of the hippocampal complex are prominent during awake states, especially when animals are involved in a cognitive task.[30] We recently reported that neonatal ketamine exposure caused elevated gamma oscillations in the subiculum in both N1 and N2 stages of NREM sleep in adolescent rats.[24] While coupling of gamma oscillations to slow oscillations is necessary for proper cognitive processing, increases in gamma during NREM may not necessarily be beneficial and we proposed that it may have contributed to learning/memory deficit in ketamine-exposed rats in Radial Arm Maze testing.[7] By contrast, this study did not find lasting abnormalities in gamma range frequency. However, we discovered that low-frequency delta (1–4 Hz) and medium-frequency sigma (9–15 Hz) oscillations are diminished in N1 stage of NREM sleep in adolescent rats that were exposed to neonatal 3β-OH when compared to vehicle-treated group. Strikingly similar phenotype of sleep oscillations in NREM stage was reported in mice with global deletion of $Ca_V3.1$ isoform of T-type channels which showed loss of thalamic delta (1–4 Hz) waves and reduction of sleep spindles (7–14 Hz) oscillations.[20] Indeed, our previous studies have shown that 3β-OH inhibits $Ca_V3.1$-mediated T-currents in the thalamic and subicular neurons in acute brain slices from neonatal rats.[7,21] However, unlike mice with global deletion of $Ca_V3.1$ channels that showed sleep fragmentation,[20] we did not observe any abnormalities in sleep macrostructure in mice exposed to 3β-OH. At the moment, the clinical significance of our findings in this study is not obvious. Although it is possible that cognitive functions may be impaired due to diminished delta and sigma oscillations in the subiculum, our previous study found that learning/memory was not affected when 3β-OH was compared *vis-à-vis* ketamine exposure using Radial Arm Maze testing.[7] Furthermore, we used *ex vivo* studies of synaptic plasticity in the subiculum to independently probe for any impairment in learning/memory in the cohorts exposed to 3β-OH and ketamine. We found in this study that the LTP induction at the CA1 → subiculum synapse was not suppressed even in the condition of artificially inducing TBS, which implies that early exposure to 3β-OH, unlike ketamine,[24] did not permanently perturb the neuronal circuitry involved in cognitive functions.

Our recent studies have documented that the hypnotic effect and cortical EEG waveforms after single administration of 3β-OH in adult rats are strongly sex-specific with females being more sensitive than males.[31] In contrast, we did not observe sex-specific differences in hypnotic effects of 3β-OH in rat pups after single and repeated injections.[7] This study was not designed to examine the possibility that lasting effects on subicular oscillations *in vivo* after repeated injections of 3β-OH in neonatal period may also be sex-specific. Furthermore, in this study, we did not interrogate any possible maladaptive changes in oscillations of thalamocortical circuit after neonatal exposure to 3β-OH. This could be an important area of future investigations since thalamocortical circuits are heavily implicated in wake-sleep cycle and are chronically hyperexcitable following exposure to common anesthetics like isoflurane and N_2O during the early stages of brain development.[32,33]

We conclude that exposure to different sedative/hypnotic agents during a critical period of brain development may induce distinct functional changes in the subiculum circuitry that may persist into adolescent age. More extensive behavioral studies are needed to compare performance of various cognitive tasks of adult rodents exposed to neonatal neuroactive steroids or ketamine and possibly other commonly used clinical drugs with different mechanisms of action (e.g. propofol).

AUTHORS' CONTRIBUTIONS

BF-R, FMM, SMJ., JEO., RMD., DS, VT, NA, and YHR performed experiments and analyzed the data. DFC provided 3β-OH. VJ-T, PSH, and SMT designed the studies, supervised the overall project, and performed final manuscript preparation.

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