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Reduced sleep and impaired sleep initiation in adult male rats exposed to alcohol during early postnatal period

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Abstract

Prenatal alcohol exposure (AE) is associated with cognitive and neurobehavioral abnormalities, such as increased motor activity and elevated anxiety, that may last a lifetime. Persistent sleep disruption may underlie these problems. Using a rat model, we investigated long-term alterations of sleep-wake behavior following AE during a critical early developmental period. Male rats received 2.6 g/kg of alcohol intragastrically twice daily on postnatal days (PD) 4-9, a developmental period equivalent to the third trimester of human pregnancy (AE group), or were sham-intubated (S group). On PD52-80, they were instrumented for tethered electroencephalogram and nuchal electromyogram recording and habituated to the recording procedures. Sleep-wake behavior was then recorded during one 24 h-long session. Wake, slow-wave sleep (SWS) and rapid eye movement sleep (REMS) were scored in 10 s epochs during 6 h of the lights-on (rest) and 6 h of the lights-off (active) periods. During the active period, REMS percentage was significantly lower (4.7±0.9 (SE) *vs.* 8.2±0.9; $p<0.02$) and the percentage of SWS tended to be lower ($p=0.07$) in AE than S rats (N=6/group). During the rest period, sleep and wake amounts did not differ between the groups, but AE rats had longer latency to both SWS and REMS onset ($p=0.02$ and 0.003, respectively). Our data demonstrate that, in a rat model of prenatal AE, impaired sleep-wake behavior persists into the adulthood. Disordered sleep may exacerbate cognitive and behavioral disorders seen in human victims of prenatal AE.

Keywords

development; fetal alcohol spectrum disorders; GABA; hypothalamus; sleep

Prenatal alcohol exposure (AE) is an established cause of disrupted sleep and abnormal sleep electroencephalogram (EEG) in infants. These disruptions correlate with subsequent delays in neurocognitive development [1,2]. Prenatal AE also is a key predictor of disrupted sleep in older children [3] which, in turn, may adversely affect development of cognitive functions and behavior [4]. It is therefore plausible that long-term cognitive and behavioral consequences of prenatal AE may be secondary to, or exacerbated by, sleep deficits. Although results of previous animal studies generally support the concept of long-term consequences of prenatal AE on sleep-wake behavior [5–7], the nature of sleep deficits and the conditions of AE sufficient to produce them are still uncertain, which complicates any

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systematic investigation of the underlying mechanisms. To date, the effects of early developmental AE on the 24-h sleep pattern were studied with only a short (2 days) and moderate prenatal AE. The result was a reduction of rapid eye movement sleep (REMS) in adult female rats and a similar trend in male rats, with no changes in slow-wave sleep (SWS) [6]. In contrast, in another study with chronic prenatal AE on gestational days 8 through 20, SWS was significantly increased in adult male rats [7].

Studies in humans and animal models suggest that the nature of different fetal alcohol effects vary with the timing and pattern of AE [1,8–10]. A later gestational exposure appears to have stronger effects on the development of motor activity and cognitive functions than earlier exposures [9,10]. In addition, binge-like AE which produces intermittently high blood alcohol concentrations (BAC) appears to be more damaging to the brain, including the regulation of sleep, than persistently elevated BAC [1,8]. These effects are well reproduced in a neonatal rat model in which AE occurs during a period of brain growth spurt equivalent to the period of human development during the third trimester of pregnancy [8,10–12]. In this model, AE is adjusted daily based on the body weight of each pup, which allows one to precisely control alcohol dosage and timing of administration. We chose this well-established model to better characterize the sleep phenotype in adult male rats exposed early in their development to alcohol and to test the hypothesis that AE during the period of brain development equivalent to the third trimester of human gestation leads to a long-lasting dysregulation of sleep-wake behavior. A preliminary report has been published [13].

All animal procedures followed the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Experiments were performed on male Sprague-Dawley rats. The animals were housed on a 12:12 light/dark schedule with lights on at 7 am and *ad libitum* access to food and water. Pups were obtained from nine timed-pregnant rats with the date of birth designated as postnatal day (PD)0. Male pups were cross-fostered to create four experimental litters with 9–10 pups, with each litter containing pups obtained from different mothers. On PD4, the litters were randomly assigned to either alcohol- (AE) or sham-treated (S) group (non-mixed design [11]). On PD4 through PD9, the AE group was treated with alcohol administered in two intragastric intubations per day (2.625 g/kg per intubation, 11.9% v/v in a custom milk formula, 2 h apart), as described previously [14]. An additional intubation with milk only was given to the AE group 2 h after the second alcohol administration. The S group was subjected to the same daily routine of intubations, but no fluid was infused in order to prevent accelerated growth of S animals due to increased caloric intake [12]. The pups were weighed prior to each intubation and were returned to the dams after each intubation. During the treatment, body weight gains remained significantly lower in the AE group, as we described previously [14]. On PD4, trunk blood was collected 2 h after the second intubation from a separate group of 10 AE rats killed by decapitation. Blood alcohol concentration (BAC) was determined using NAD-ADH reagent (Sigma, St Louis, MO, USA). The mean BAC was 334±19 (SE) mg/dl, which was consistent with previous studies with a similar design [10,14]. The offspring were weaned on PD21-22.

On PD52-80, the rats were instrumented for recording of cortical EEG and nuchal electromyogram (EMG). All surgical procedures were performed under aseptic conditions, as described previously [15]. Briefly, the animals were anesthetized with i.m. ketamine (87 mg/kg) and xylazine (13 mg/kg) followed by isoflurane (1–2.5%). The frontal and parietal bones were exposed, and three stainless steel screws were inserted for EEG recording from the frontal cortex and for electrical reference. A pair of stainless steel, multi-stranded wires was sutured into the dorsal nuchal musculature to record the EMG. Following a recovery

period of at least 7 days, the rats were habituated to the handling, hook-up procedure, and recording chamber during 2–6 h each day for two days and then for 10–12 h during an overnight session. The habituation sessions were separated by at least 24 h. Sleep-wake behavior was recorded during habituation sessions to verify the integrity of the connections and quality of the signals. During all recordings, the rat was placed in its home cage and connected with a counter-weighted cable and a swivel (SL6C, Plastics One, Roanoke, VA, USA) to amplifiers (Electroencephalograph model 8–10, Grass, Quincy, MA, USA). The recording cage was placed in a sound-dampened and electromagnetically shielded chamber (1 m^3). EEG and EMG signals were initially filtered (0.3–70 Hz and 5–10,000 Hz, respectively) and then digitized at 100 Hz for the EEG and 1000 Hz for the EMG and stored on a hard disk (Spike-2 software, Cambridge Electronic Design Ltd., Cambridge, UK). Animal movements were counted using infrared beam breaking technique (MicroMax, AccuScan Instruments, Columbus, OH, USA). At the time of final sleep-wake recording, the mean body weight of AE rats did not differ from that of S rats (391 g±17 (SE) *vs.* 386 g±11, respectively). Three of nine AE rats and two of eight S rats that were instrumented had signals of unsatisfactory quality and were excluded from the study.

On PD70-97, undisturbed sleep-wake behavior of each rat was recorded during one 24 h-long session that started at 10 AM. Three behavioral states, wake, SWS and REMS, were distinguished in successive 10 s epochs using standard criteria based on the appearance of the cortical EEG and nuchal EMG and the EEG power spectrum simultaneously displayed for each scoring epoch (Somnologica v. 3, Medcare/Embla, Buffalo, NY, USA). The scoring was conducted by a person blind to the treatment and its accuracy was verified as described previously [15].

The following measures were obtained: latency from the hook-up time to the first episode of SWS and REMS, percentage of recording time spent in each behavioral state, and the number and duration of wake, SWS and REMS episodes. For comparison between AE and S rats, all parameters other than sleep onset latency were calculated over a period of last 6 h of recording during the lights-on period (12 PM-6 PM) and last 6 h of the lights-off period (12 AM-6 AM). To characterize motor activity, the total activity (total number of beam interruptions) and the number of movement bouts (number of separate periods during which the animal continuously crossed different sets of beams with breaks between such periods longer than 1 s) were quantified using Microlyze software (AccuScan Instruments).

All datasets were tested for normality and equal variance. Statistical significance of differences between the treatment groups and recording periods was then tested using one-way analysis of variance (ANOVA) with Bonferroni's correction. When normality criteria were not met (SWS onset, number of REMS episodes (lights-on period), number of movement bouts (lights-off period) for the AE group, and duration of wake (lights-off period) and REMS episodes (lights-on period) for the S group), Kruskal-Wallis ANOVA with Bonferroni's correction was used. The Pearson's correlation was used to determine the relationship between the percentages of sleep and the total motor activity. Differences were regarded significant at $p<0.05$ (Analyse-It software, Leeds, UK).

The AE and S rats had similar circadian patterns of sleep-wake behavior and motor activity, with the characteristically higher amounts of sleep during the lights-on period and lower amounts during the lights-off period ($p=0.048$–0.0001 for day-night differences in the percentage and number of episodes of wake, SWS and REMS, total motor activity and number of movement bouts). In both groups, the percentages of both SWS and REMS were inversely proportional to the total motor activity across the 24 h (r −0.75 to −0.97, $p<0.0001$). Superimposed on this general pattern were significantly reduced amounts of sleep in AE rats near the beginning and the end of the lights-off period (Fig. 1A). In

addition, the latencies to the onset of the first episodes of both SWS and REMS were higher in AE than in S rats (p=0.02 and 0.003, respectively; Fig. 1B).

During the last 6 h of the lights-off periods, AE rats had more wakefulness (p=0.029), lower percentage of REMS (p=0.017) and a tendency for reduced amount of SWS (p=0.07) when compared to S rats (Fig 2, right side of the graphs). Both the number and duration of individual sleep episodes tended to be lower in AE than S rats, but the differences were not significant (Fig. 3A–D, right side of the graphs), whereas the duration of wake episodes tended to increase (4.96 min ±0.71 (SE) *vs.* 3.48±0.41 in S group, p=0.15; not shown). Consistent with an increased amount of wakefulness, motor activity tended to be higher in AE than S rats during the lights-off period (Fig. 3E,F), but the trend was absented when motor activity was scaled by the amount of wakefulness (not shown). Thus, AE rats did not exhibit genuine hyperactivity. In contrast to the lights-off period, there were no significant differences other than in the sleep onset latency between the study groups during the lights-on period (Figs. 2–3, left side of the graphs).

Our data show that adult male rats exposed to alcohol during the developmental period equivalent to the third trimester of human gestation have reduced sleep amounts during the active period and difficulty to initiate sleep during the rest period. We found that, during the lights-off period, AE rats had significantly increased amount of wakefulness at the expense of significantly reduced amount of REMS and, to a lesser extent, SWS. A significant reduction of REMS following prenatal AE was previously reported for female rats but was not significant in male rats [6]. However, the design of that study was different from ours in that it utilized a less severe, indirect alcohol administration at an earlier gestational period (0.9 g of alcohol per kg of pregnant female's body weight on gestational days 7 and 8).

We did not use a non-intubated control group [12]. However, any effects of intubation *per se* would apply to both AE and S group and, therefore, be controlled for in the comparisons between the groups. Studies with a more complex design and larger study groups would be needed to determine whether an interaction between the effect of neonatal intubation and treatment has a differential effect on sleep-wake behavior of adult rats.

A recent study indicated that adult rats that were prenatally exposed to alcohol exhibit anxiety-like behavior due to deficient inhibition mediated by γ-aminobutyric acid (GABA) in the basolateral amygdala [16]. Indeed, altered GABAergic mechanism not only in the amygdala but also other regions important for the control of vigilance and emotions may underlie disordered sleep initiation and maintenance in victims of prenatal AE. We earlier reported that immature rats subjected to AE on PD4-9 have reduced levels of enzymatic GABA precursor, glutamate decarboxylase (GAD), in the hypothalamic sleep-regulatory sites [17]. One of these sites, the ventrolateral preoptic (VLPO) region of the anterior hypothalamus, contains GABAergic neurons that are active during sleep [18]. Lesions of the VLPO and adjacent regions reduce the amount of SWS and REMS, suggesting that these cells are necessary for the normal generation of sleep [19]. Another region where GAD levels also were reduced following early postnatal AE was the perifornical (PF) region of the posterior lateral hypothalamus which has wake-promoting functions [17]. These data suggest that early postnatal AE may disrupt development of the hypothalamic GABAergic system, thus altering important sleep-wake-controlling network.

Data indicate that the magnitude of the REMS reduction following the lesions of the VLPO nucleus and the "extended' VLPO is greater during the lights-off than the lights-on period [19]. Similarly, we found that the significant reduction of REMS following the AE occurred during the lights-off period. The preferential reduction of REMS during the lights-off period

may be related to the absence during the rest period of a circadian system-related input from the hypothalamic suprachiasmatic nucleus that facilitates transitions into REMS [20].

The posterior hypothalamus, where we previously observed changes in GAD expression following early postnatal AE, is also implicated in anxiety disorders [21]. The problems with sleep initiation and maintenance that we found in the present study may have at their root increased anxiety that was previously reported in other animal models of fetal alcohol spectrum disorders (FASD) [16]. One of the best established, long-term adverse consequences of prenatal AE is hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis which, in turn, is associated with both insomnia and increased vulnerability to depression and anxiety disorders, the most commonly diagnosed mental disorders in FASD patients [22,23]. The prevalence of disordered sleep is high in children and adolescents with anxiety disorders [24]. Importantly, insufficient sleep is now seen not only as a symptom but also as one of the causes of depression [25]. Thus, our data emphasize the need for further assessment of the mechanisms and impact of disordered sleep in FASD. If sleep disruption contributes as a major factor to other neurobehavioral consequences of perinatal AE, this would call for increased efforts to normalize sleep in victims of prenatal AE as a preventive strategy that may improve other symptoms. Since our data suggest that our model robustly replicates long-term sleep disorders typical of FASD, the model should prove useful for investigation of the role of sleep disruption in the development of cognitive and affective disorders associated with FASD.

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ABBREVIATIONS

AE	alcohol exposure
ANOVA	analysis of variance
BAC	blood alcohol concentration
EEG	electroencephalogram
EMG	electromyogram
FASD	fetal alcohol spectrum disorders
GABA	γ-aminobutyric acid
GAD	glutamate decarboxylase
HPA	hypothalamic-pituitary-adrenal
PD	postnatal day
PF	perifornical
REMS	rapid eye movement sleep
S	sham-treated
SE	standard error
SWS	slow-wave sleep
VLPO	ventrolateral preoptic

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Highlights

- Rats received alcohol intragastrically or were sham-intubated on postnatal days 4–9.
- 24 h recordings of sleep-wake behavior were obtained when the rats reached adulthood.
- Alcohol-exposed rats had longer latency to sleep onset during the rest period.
- Alcohol-exposed rats had less rapid eye movement sleep during the active period.
- Disrupted sleep may exacerbate disorders associated with prenatal alcohol exposure.

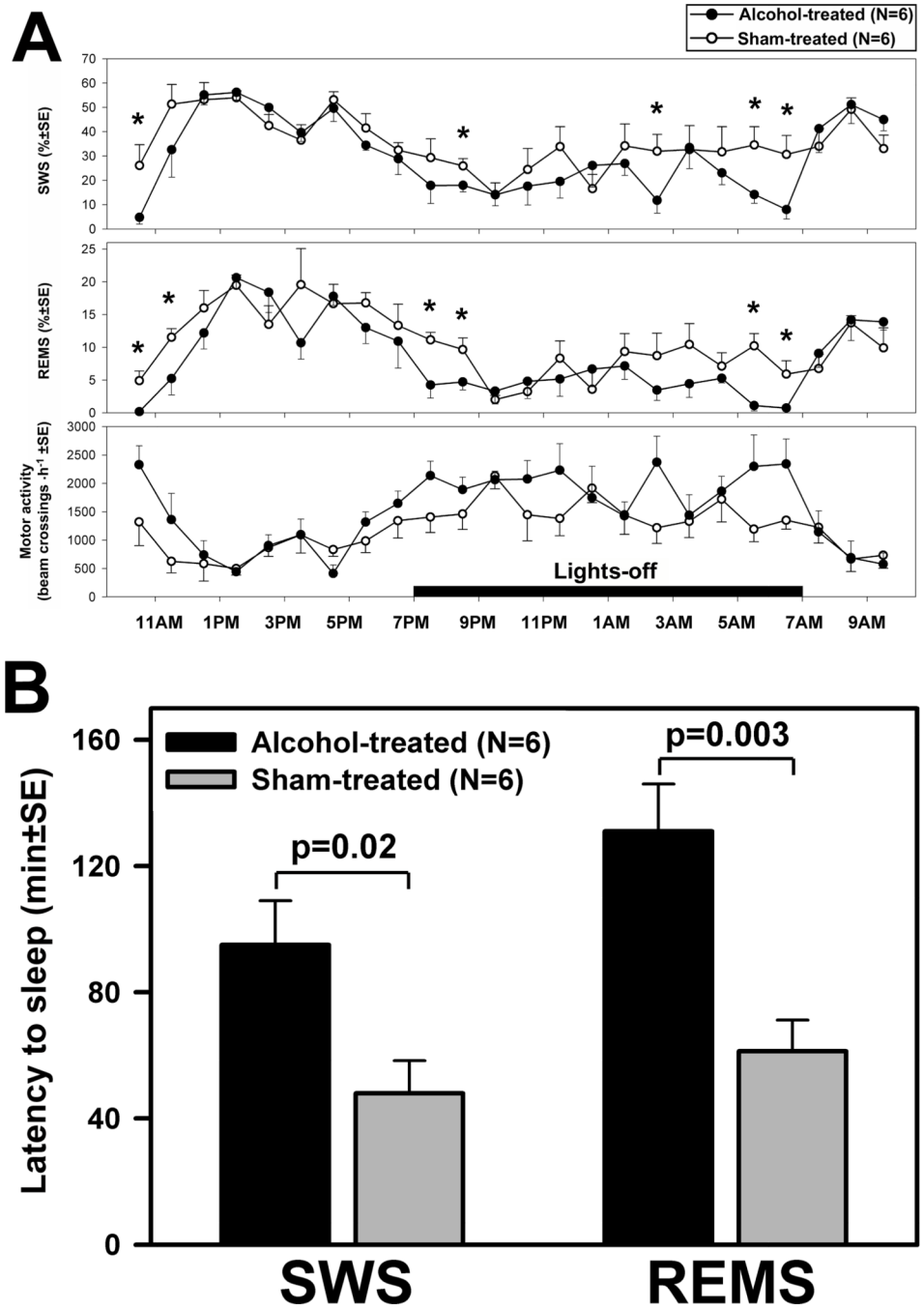

Fig. 1.

Effect of early postnatal alcohol exposure on sleep and motor activity in adult male rats (N=6 per group). A: average time course of slow-wave sleep (SWS, top panel), rapid eye movement sleep (REMS, middle panel) and motor activity (bottom panel) in AE and sham-treated (S) rats measured in successive 1 h intervals over 24 h period. Both SWS and REMS were significantly reduced in AE rats at the beginning and near the end of the lights-off period (black bar along the time axis). B: mean latencies to the first episode of SWS and REMS were longer in AE than S rats.

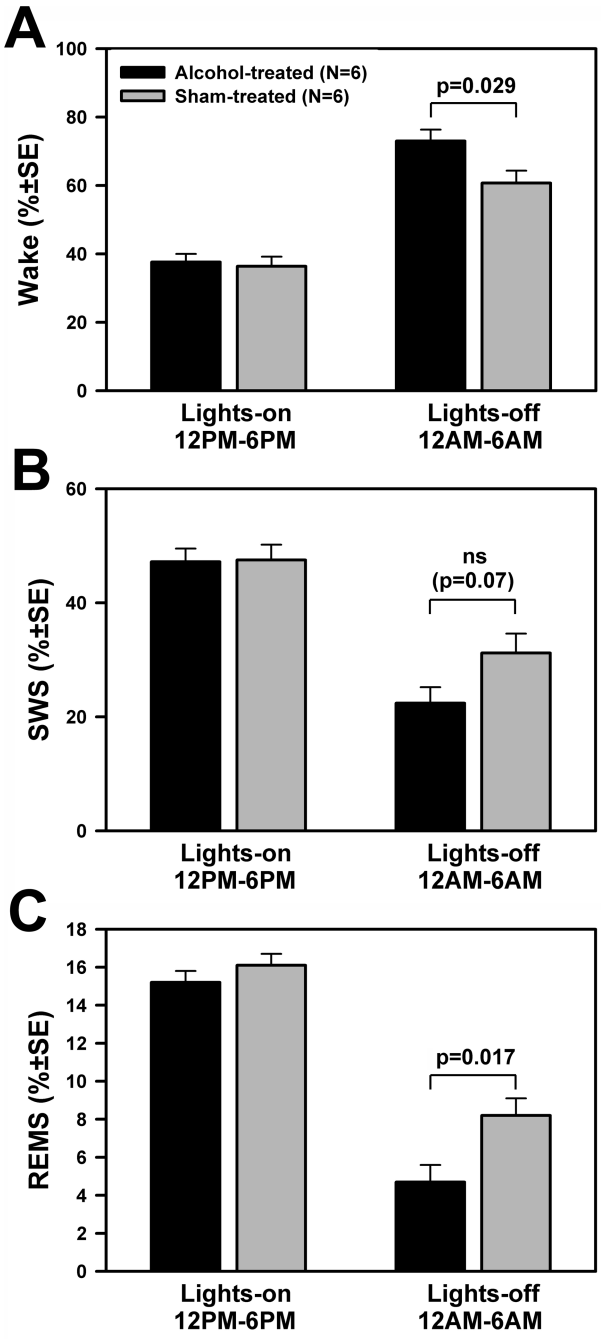

Fig. 2.

Effect of early postnatal alcohol exposure on the percentage of wake (A), SWS (B) and REMS (C) measured during the last 6 h of the lights-on and the last 6 h of lights-off periods. The numbers of animals per group (N) and the legend for group coding in A apply to all panels. ns – not statistically significant.

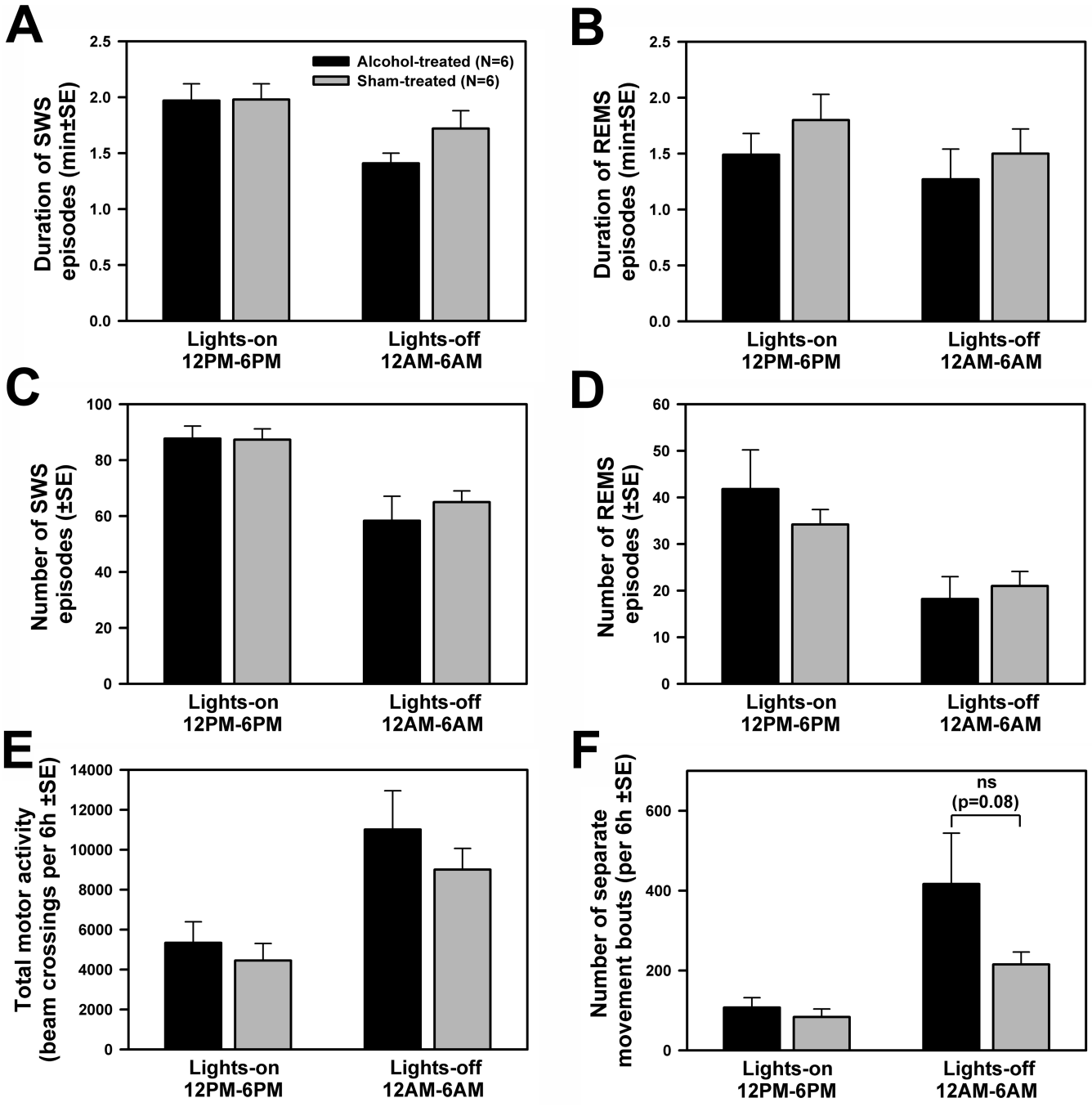

Fig. 3.

Effect of early postnatal alcohol exposure on the number of episodes and mean episode duration of SWS (A,C) and REMS (B,D), and motor activity (E,F). E: total number of infrared beam crossings (total motor activity). F: number of movement bouts (periods during which the animal continuously crossed different sets of beams with breaks between bouts longer than 1 s). The numbers of animals per group (N) and the legend for group coding in A apply to all panels. ns – not statistically significant.