Prenatal nicotine alters vigilance states and AchR gene expression in the neonatal rat: implications for SIDS

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Frank, Marcos G., Hilary Srere, Carlos Ledezma, Bruce O’Hara, and H. Craig Heller. Prenatal nicotine alters vigilance states and AchR gene expression in the neonatal rat: implications for SIDS. Am J Physiol Regulatory Integrative Comp Physiol 280: R1134–R1140, 2001.—Maternal smoking is a major risk factor for sudden infant death syndrome (SIDS). The mechanisms by which cigarette smoke predisposes infants to SIDS are not known. We examined the effects of prenatal nicotine exposure on sleep/wake ontogeny in the neonatal rat. Prenatal nicotine exposure transiently increased sleep continuity and accelerated sleep/wake ontogeny in the neonatal rat. Prenatal nicotine also upregulated nicotinic and muscarinic cholinergic receptor mRNAs in brain regions involved in regulating vigilance states. These findings suggest that the nicotine contained in cigarette smoke may predispose human infants to SIDS by interfering with the normal maturation of sleep and wake.

METHODS

Nicotine administration. We duplicated protocols designed to mimic nicotine exposure levels in human fetuses whose mothers smoke during pregnancy (35). Timed pregnant Sprague-Dawley rats were obtained from Simonsen laboratories on the 2nd to 3rd gestational day (G2-G3) and were housed in our rat colony (Ta 22C: 12:12-h light-dark schedule) and were provided rat chow and water ad libitum. On G4-G5, the pregnant dams were anesthetized with halothane (1.5%–2.5%) and subcutaneously implanted with type 2ML2 OSMI minipumps filled with nicotine (0.1 mg/mL) and stored in a constant temperature cabinet at 37°C. Nicotine was delivered at a rate of 1 μg/h to a group of pregnant rats and at a rate of 0.05 μg/h to a group of control rats. The minipumps remained in the rats until birth. The litter size was 8–10 pups per dam. One week prior to birth, the minipumps were removed and the rats were housed alone (2 weeks) to mimic the isolation experienced by SIDS infants. The rats were then returned to the pregnant dam and allowed to recover for 1 week. All maternal and neonatal procedures were performed as described previously (10, 11).

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with nicotine bitartrate (in 1 of 2 doses: 17 or 35 mg/ml in bacteriostatic water) or vehicle (VEH: sodium bitartrate 20 mg/ml in bacteriostatic water). On the basis of final gestational weights of 350 g, the two nicotine concentrations deliver 2 (NIC2) and 4 mg·kg⁻¹·day⁻¹ (NIC4), respectively, by the end of the infusion period (35). The pumps were removed at parturition (P0). Three to four litters were used for each group, and no more than two male pups were used from any one litter.

**Neonatal rat EEG and EMG Implantation.** On the 10th postnatal day (P10), male pups from each of the three groups (VEH: n = 6; NIC2: n = 7; NIC4: n = 7) were prepared for EEG/EMG recording according to previously described methods (10, 11). Briefly, pups were placed on a heating pad and anesthetized with methoxyflurane inhalant. Miniaturized EEG electrodes (#000 stainless steel screws) were implanted bilaterally over frontal and parietal cortex. EMG electrodes (braided stainless steel, insulated wire) were bilaterally inserted into nuchal muscles. On P11, the pups were fitted with stainless steel feeding cannulae. The Stanford University Administrative Panel for Laboratory Animal Care approved all surgical procedures.

**Neonatal rat housing procedures.** Twelve hours before the first sleep registrations (at 2000), pups from the three groups were attached to flexible recording cables that were in turn connected to slip-ring commutators. Feeding tubes were attached to the cheek cannula and connected to an infusion pump. Pups were then placed in acrylic incubators containing home bedding material and were warmed by a self-enclosed water bath housed in a grounded Faraday cage. The incubator temperatures were adjusted at each age to compensate for increasing thermogenesis in the rat pups (10, 11). P12 and P16 rats were periodically groomed and were fed an enriched milk formula according to schedules previously shown to provide for normal growth and to control for the effects of maternal separation on sleep organization (10, 11). P20 and P24 rat pups were provided with water and rat chow ad libitum. All sleep registrations were made on the next day at 0800 (lights on in a 12:12-h light-dark cycle) and continued for 24 h. At the end of each sleep/wake registration (at P13, P17, P21, and P25, respectively), the pups were disconnected from the feeding and recording equipment, weighed, and returned to their home nests.

**Neonatal sleep recordings and vigilance state analyses.** EEGs and EMGs were recorded on a Grass 7 polygraph. Unihemispheric, frontal-parietal EEG potentials were band-pass filtered at 0.3 and 35 Hz (one-half maximum, 6 dB/octave), digitized at 100 Hz, and stored in 10-s epochs on a personal computer. EMG signals were full-wave rectified, integrated, and stored as one value per epoch. The EEG was then Fourier analyzed in each 10-s epoch by use of a Fast Hartley Transform. The digitized polygraphic records were then inspected epoch by epoch, and artifacts (on average, 90% agreement with polygraphic scoring) was used to determine vigilance states (11). Low levels of EEG δ-power coupled to EMG minima characterized rapid eye movement (REM) sleep (REM sleep is used to refer to all EEG-defined REM sleep-like states in this investigation, because current evidence suggests that REM sleep is present in P12 rats (6)). High levels of EEG δ-power (0.5–4.0 Hz) coupled to low and intermediate EMG values characterized SWS. Low levels of EEG δ-power coupled to intermediate and high EMG values characterized wake.

We made several measurements of sleep and wake for each group at each time point. We first calculated the amounts of REM sleep and SWS [as %total recording time (TRT) and total sleep time (TST)] and wake and total sleep (REM sleep + SWS) as percent TRT in each group. We then examined REM sleep, SWS, and wake continuity by measuring the average duration of sleep and wake bouts (in minutes). A bout was defined as a sustained vigilance state of at least 20 s, not interrupted by the occurrence of any other vigilance state. We also measured mean latencies to REM sleep (in minutes) in each group. REM sleep latencies were defined as the number of SWS minutes elapsed before each REM sleep bout of at least 20 s duration (calculating REM sleep latency by use of both SWS + W minutes elapsed produced similar results). The distribution of spectral power in sleep EEGs was analyzed by measuring SWS δ (0.5–4.0 Hz/θ) (12.0–15.0 Hz) EEG ratios and REM sleep θ (4.75–7.0)/θ ratios from P12–P24. We also measured the diurnal distribution of SWS δ-power by analyzing the hourly change in mean SWS δ-power (normalized to the 24-h mean) across the light phase for each group from P12 to P24.

All statistics were performed with SAS software. Repeated-measures ANOVAs were used to test for significant main effects (age and condition) and interactions between main effects. Ryan-Einot-Gabriel-Welsch (REGW) protected t-tests were used for further comparisons when significant main or interaction effects were obtained by the ANOVA. Student’s t-tests were used when no more than two to three comparisons were made between or within groups.

**Molecular analyses.** Male siblings from each treatment group (as described above) were killed at P12, P16, P20, and P24 to assess changes in nicotinic and muscarinic mRNAs by use of Northern blot analyses as previously described (25). Briefly, brain tissue (n = 5 males per group) was flash frozen on dry ice and then stored at −70°C until RNA isolation. At least three different litters contributed to each treatment condition and developmental time point sample. Total RNA was isolated from the tissue with TRIzol reagent (GIBCO BRL). nAChR subunit cDNAs for a4, a7, and β2 were obtained from Dr. T. Bonner (National Institute of Mental Health). Quantification of Northern blots was done using phosphorimage analysis and molecular analyst software (version 2.1, Bio-Rad). Data for all probes were normalized to glyceraldehyde-3-phosphate dehydrogenase (GPDH) mRNA levels in the same samples to correct for any variations in loading or transfer. GPDH levels appear to remain constant across all nicotine treatment conditions but may vary across age. Therefore, all statistical comparisons are across nicotine treatment groups within each age cohort. Because the relative mRNA data were not distributed normally, Mann-Whitney (two-tailed) tests and post hoc Duncan’s tests were used to assess differences in mRNA levels between groups at each time point.

**RESULTS**

**Sleep/wake architecture.** Prenatal nicotine treatments produced both transient and long-lasting effects on sleep/wake ontogenesis in the neonatal rat. Across development (P12–P24), neonatal rats prenatally exposed to nicotine had less total sleep and more wake (main effect: \( F = 13.72, P < 0.0001 \)) than rats prenatally exposed to vehicle (Table 1). The lower amounts of total sleep in the nicotine-exposed rats reflected an overall reduction of REM sleep (as %total recording time and as %total sleep time) across development (main effect: \( F = 6.55, P < 0.0003 \)) and a reduction of...
total sleep in the dark phase from P16-P24 (Table 1 and Fig. 3, D-F). Prenatal nicotine also increased the duration of wake bouts (main effect: $F = 14.7, P < 0.0001$) and REM sleep latencies (main effect: $F = 28, P < 0.0001$) across development in rats prenatally exposed to nicotine (Table 1, Fig. 1F, and Fig. 2B).

Prenatal nicotine exposure transiently increased sleep continuity in the neonatal rat. Although prenatal nicotine had no main effects on REM sleep (main effect: $F = 0.39, P = 0.76$) or SWS (main effect: $F = 2.46, P = 0.064$) bout lengths there was a significant interaction between REM sleep ($F = 3.48, P < 0.0005$) and SWS ($F = 2.57, P < 0.009$) bout length and postnatal age (Fig. 1, D and E). Neonatal rats exposed to nicotine had longer episodes (in minutes) of REM sleep and SWS at P12 (Fig. 1, D and E; REGW, $P < 0.05$) compared with control rat values.

**Sleep EEGs.** The effects of prenatal nicotine exposure on sleep EEGs were restricted to REM sleep. Neonatal rats prenatally exposed to nicotine had greater levels of EEG $\theta$ (4.75–7.0 Hz)-power (as measured by the REM sleep EEG $\theta/d$ ratio) than neonatal rats prenatally exposed to vehicle (main effect: $F = 12.06, P < 0.002$, Fig. 2A). Prenatal nicotine had no effect on SWS EEG $d/s$ power ratios (data not shown).

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**Table 1. Effects of prenatal nicotine on vigilance states, amounts, bout durations, and latency to REM sleep averaged across development 12–24 days postparturition**

<table>
<thead>
<tr>
<th></th>
<th>Wake %TRT</th>
<th>T Sleep %TRT</th>
<th>REM Sleep Latency, min</th>
<th>REM Sleep, %TRT, %TST</th>
<th>SWS %TRT, %TST</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEH</td>
<td>31.5 ± 1.3</td>
<td>68.5 ± 1.3</td>
<td>5.17 ± 0.53</td>
<td>33.8 ± 2</td>
<td>34.8 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>[2.0 ± 0.2]</td>
<td>[1.9 ± 0.1]</td>
<td>(47.7 ± 2.4)</td>
<td>(52.26 ± 2.4)</td>
<td></td>
</tr>
<tr>
<td>NIC2</td>
<td>35.2 ± 1.4*</td>
<td>64.8 ± 1.4*</td>
<td>7.22 ± 0.84*</td>
<td>29.0 ± 1.9*</td>
<td>35.8 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>[2.7 ± 0.3]*</td>
<td>[2.0 ± 0.1]</td>
<td>(42.6 ± 2.2)</td>
<td>(57.4 ± 2.2)</td>
<td></td>
</tr>
<tr>
<td>NIC4</td>
<td>35.2 ± 1.2*</td>
<td>64.8 ± 1.3*</td>
<td>7.78 ± 0.86*</td>
<td>28.6 ± 2.0*</td>
<td>36.3 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>[3.1 ± 0.3]*</td>
<td>[2.1 ± 0.1]</td>
<td>(42.9 ± 2.7)*</td>
<td>(57.1 ± 2.7)</td>
<td></td>
</tr>
</tbody>
</table>

Data represent 24-h means ± SE of wake, total (T) sleep, rapid eye movement (REM) sleep, and slow-wave sleep (SWS) amounts, expressed as %total recording time (TRT) and %total sleep time (TST). Values in brackets represent means ± SE of durations of wake, REM sleep, and SWS bouts in minutes. VEH, vehicle; NIC2 and NIC4, 2 and 4 mg nicotine-kg$^{-1}$-day$^{-1}$, respectively. *Significant difference from VEH values (ANOVA, Ryan-Einot-Gabriel-Welsch, $P < 0.05$).

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**Fig. 1. Effects of prenatal nicotine on sleep/wake ontogenesis from day 12 postparturition (P12) to P24**. VEH, vehicle; NIC2 and NIC4, 2 and 4 mg nicotine-kg$^{-1}$-day$^{-1}$, respectively. A–C: mean 24-h (± SE) rapid eye movement (REM) sleep, slow-wave sleep (SWS), and wake amounts expressed as %total recording time (TRT). D–F: mean 24-h (± SE) REM sleep, SWS, and wake bout durations from P12–P24. There were no significant light-dark phase differences in these measurements (within states) except as indicated in Fig. 3. *Significant difference between NIC2, NIC4 vs. VEH values; **significant difference between NIC4 vs. VEH values. [Ryan-Einot-Gabriel-Welsch (REGW) protected t-tests, $P < 0.05$].

**Fig. 2. Effects of prenatal nicotine on REM sleep.** A: prenatal nicotine increased EEG $\theta$ (4.75–7.0 Hz)-activity in neonatal rats. Data represent mean 24-h (± SE) REM sleep EEG theta expressed as a $\theta/d$ ratio. B: prenatal nicotine increases latency to REM sleep. Data represent mean 24-h REM sleep latencies in minutes (±SE). *Significant difference between NIC2, NIC4 vs. VEH values; **significant difference between NIC2 vs. VEH values; ***significant difference between NIC4 vs. VEH values (REGW, $P < 0.05$).
Twenty-four hour organization of sleep/wake states. Prenatal nicotine produced an early appearance of 24-h organization of sleep and wake (Fig. 3). Neonatal rats prenatally exposed to nicotine had more wake and less total sleep (as %total recording time) and longer wake bouts (in minutes) in the dark phase compared with the light phase by P16–P20. Neonatal rats treated with vehicle did not show similar 24-h organization in wake until P24 (Student’s t-test, P < 0.05). REM sleep and SWS bout durations were not significantly longer in the light phase compared with the dark phase at any age in the nicotine- and vehicle-treated groups (data not shown).

We also examined the distribution of SWS EEG δ-power across the rest phase in all groups. A peak and subsequent decrease in SWS EEG δ-power during the rest phase is typical of adult mammals, reflecting the homeostatic accumulation and discharge of sleep need (2). We observed an adultlike decrease in SWS EEG δ-power (0.5–4.0 Hz) across the light phase in NIC4 rats at P24 but not in rats receiving the lower dose of nicotine or vehicle (Fig. 3, G–I).

Molecular results. In agreement with a previous study (32), we found that prenatal nicotine increased the expression of AchR mRNAs in the neonatal rat brain. These effects were chiefly confined to the NIC4 group and varied across brain region and postnatal age (Table 2). In the NIC4 group, for example, nAChR α4 mRNAs were elevated only in the thalamus (from P16 to P24), whereas nAChR α7 mRNAs were elevated in the thalamus (P16), hypothalamus (P12–P24), and basal forebrain (P16–P20). Smaller changes in nAChR mRNAs were observed in the NIC2 group and reached significance only in the basal forebrain at P12 (P < 0.001). Prenatal nicotine had similar effects on m1 AchR mRNA expression. m1 AchR mRNAs were elevated in the basal forebrain at P12 and P20 in the NIC2 group (P < 0.01) and from P16–P20 in the NIC4 group (P < 0.001). The effects of prenatal nicotine on m1 AchR mRNAs in the thalamus and hypothalamus could not be determined, because amounts were below quantitative levels in our Northern analyses (data not shown).

General maturation. Prenatal nicotine did not impair normal growth rates in our neonatal rats. The mean body mass of all groups at weaning (P20) was similar (NIC2: 39.5 ± 1.7; NIC4: 40.5 ± 1.8; VEH: 34.5 ± 2.3 g) and eye opening began in all groups at approximately P13.

DISCUSSION

We investigated the effects of prenatal nicotine on sleep/wake ontogenesis and the expression of central cholinergic receptor mRNAs in the neonatal rat. We found that prenatal nicotine profoundly altered sleep/
wake ontogenesis and upregulated central cholinergic mRNAs in several brain regions that regulate sleep and wake. These findings and their implications for SIDS are discussed below.

**Nicotine and the developing nervous system.** An abnormally rapid maturation of sleep and wake in rats prenatally exposed to nicotine is consistent with nicotine’s teratogenic effects on the developing nervous system. Nicotine mimics the trophic functions of acetylcholine, causing premature cell differentiation in the developing brain (24, 35). The results of this premature trophic signaling on the brain are wide ranging, including changes in cholinergic (24, 33, 38) and monoaminergic (26, 35) systems that regulate sleep and wake. Prenatal nicotine, for example, has multiple teratogenic effects on the cholinergic system (24, 33, 35, 38) that are likely to affect wake and REM sleep, since cholinergic neurotransmission plays an essential role in these vigilance states (19, 31, 36).

The developing circadian system may also be affected by the trophic actions of prenatal nicotine. The SCN contains an endogenous oscillator that distributes the amount of sleep and wake across the 24-h day (7). The fetal SCN may be particularly sensitive to the trophic actions of nicotine during the latter half of gestation (3, 25). An abnormally rapid differentiation of SCN cells could result in precocious circadian organization in sleep/wake expression and more consolidated wake periods, because these aspects of sleep and wake are regulated by the SCN (7).

**Prenatal nicotine and AChR mRNAs.** In agreement with an earlier study (32), we found that prenatal nicotine exposure in the rat increased nAChR a4, a7, and b2 mRNAs in several brain regions that regulate sleep and wake. We also found increases in m1 AchR mRNA in the basal forebrain of treated animals. The functional significance of these molecular findings is unclear. In the case of muscarinic receptors, previous work (33) using the same nicotine exposures found a decrease in receptor binding for m2 receptors in the brain stem and for m1 receptors in the striatum but not in the hippocampus (38). It is possible, therefore, that the upregulation we observe in m1 mRNA is compensatory to a decrease in protein. It is also possible that, in the basal forebrain, both mRNA and protein levels increase. For nAChRs, changes in mRNA, protein, and function do not typically change in concert, as is common with most mRNAs and proteins. In fact, in the adult, upregulation of nAChR after nicotine exposure is often coincident with receptor desensitization, leading to reduced responsiveness. Furthermore, this upregulation of nAChRs does not appear to involve any upregulation of the respective mRNAs (28). Therefore, it is not clear whether the upregulation we observe in nAChR mRNAs in certain brain regions during these developmental periods leads to hypo- or hyperresponsiveness to ACh. In either event, however, such changes could contribute to the observed alterations in sleep parameters, especially in light of the substantial changes AChRs undergo during development (1, 25).

**Significance for SIDS.** Maternal smoking is a major risk factor for SIDS (14, 22). Precisely how maternal smoking increases the risk of SIDS is unknown. In recent years, the nicotine contained in cigarette smoke has come under increasing scrutiny as a factor in the increased risk of SIDS in infants exposed during gestation to cigarette smoking. Neonatal rats prenatally treated with nicotine are more likely to die during a hypoxic challenge (34), and perinatal nicotine exposure reduces the ability of neonatal rats to autoresuscitate from obstructive apneas (8). In addition, human infants exposed during gestation to cigarette smoking have abnormal nAChR binding in brain stem areas important in respiration and arousal (23). These an-
mal and human studies suggest that the nicotine released into the placental blood supply from maternal smoking compromises human cardiorespiratory development, thereby increasing the risk of SIDS.

The results of the present study indicate that maternal smoking may also alter the fetal development of sleep and wake mechanisms. Prenatal nicotine exposure in the rat produced abnormalities in sleep and wake similar to those found in infants at risk for SIDS (infants exposed to SIDS risk factors, near-miss infants, and siblings of SIDS victims). For example, prenatal nicotine increased the continuity (duration) of REM sleep and SWS bouts in the youngest rats (P12). Because increases in sleep continuity are associated with deeper, more intense sleep (2), this result suggests that prenatal nicotine transiently deepens sleep in neonatal rats. This result is particularly interesting because SIDS may be caused by abnormally deep sleep periods (12, 16, 37), and human infants exposed to cigarette smoking during gestation have abnormally deep sleep (9).

We also found that sleep and wake appear to develop at an accelerated rate in neonatal rats prenatally exposed to nicotine. Prenatal nicotine reduced total sleep (primarily due to a reduction in REM sleep amounts) and increased wake, REM sleep latencies, and REM sleep EEG θ-activity. Prenatal nicotine exposure also produced a rapid development of 24-h organization in wake amounts, wake continuity, and SWS δ-power. These findings are consistent with an abnormally rapid maturation of sleep and wake in rats prenatally exposed to nicotine, because all of these changes in sleep and wake are normally observed during the course of development, but at later, postnatal dates (6).

Similar findings have been reported in infants at risk for SIDS. More consolidated wake periods, longer latencies to REM sleep, a rapid maturation of REM sleep cyclicity, and lower REM sleep amounts are reported in infants at risk for SIDS (15, 27, 37). Infants at risk for SIDS also show precocious circadian organization in sleep (30) and sleep respiratory patterns (18) and more rapid maturation in sleep EEGs compared with age-matched infants not at risk for SIDS (29, 37). Although the relationship between maturational rates and SIDS is not yet clear (4, 5, 13), our results are consistent with several findings in humans and suggest that SIDS is associated with an accelerated development of sleep and wake that may be induced or amplified by maternal smoking.

Perspectives

It has been suggested that accelerated sleep/wake ontogenesis may trigger SIDS events by producing developmental misalignments between cardiac, respiratory, and sleep systems that normally become integrated in the postnatal period (37). One consequence of such misalignment might be abnormal sleep periods in infants who, due to comparatively immature or compromised cardiorespiratory mechanisms, do not arouse during a life-threatening event such as a prolonged sleep apnea (12, 15, 37). If this hypothesis is correct, then the increased rates of SIDS in infants exposed to cigarette smoking during gestation may be related to nicotine's effects on the ontogenesis of sleep and wake.

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