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SMOKING DURING PREGNANCY: POSTNATAL EFFECTS ON AROUSAL AND ATTENTIONAL BRAIN SYSTEMS

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Abstract

Prenatal exposure to cigarette smoke is known to produce lasting arousal, attentional and cognitive deficits in humans. The pedunculo pontine nucleus (PPN), as the cholinergic arm of the reticular activating system (RAS), is known to modulate arousal, waking and REM sleep. Rapid eye movement (REM) sleep decreases between 10 and 30 days postnatally in the rat, with the greatest decrease occurring at 12–21 days. Pregnant dams were exposed to 150 ml of cigarette smoke for 15 min, 3 times per day, from day E14 until parturition, and the pups allowed to mature. We analyzed a) intrinsic membrane properties of PPN neurons in slices from pups aged 12–21 days, and b) the sleep state-dependent P13 auditory evoked potential, which is generated by PPN outputs, in animals allowed to age to adolescence. We found significant changes in the intrinsic membrane properties of PPN cells in prenatally exposed animals compared to intact ones, rendering these cells more excitable. In addition, we found disturbances in the habituation to repetitive stimulation in adolescent, freely moving animals, suggestive of a deficit in the process of sensory gating. These findings could explain some of the differences seen in individuals whose parents smoked during pregnancy, especially in terms of their hypervigilance and increased propensity for attentional deficits and cognitive/behavioral disorders.

Keywords

arousal; attention; nicotine; pedunculo pontine nucleus; rapid eye movement sleep; reticular activating system

Introduction

Nicotine (NIC) from cigarette smoke can cross into the placenta so that fetuses of mothers who smoke are exposed to higher NIC concentrations in both amniotic fluid and umbilical vein than maternal vein serum (Luck et al., 1985). Coupled with an increased propensity for young women to begin smoking during the childbearing ages of 18–25, prenatal exposure to cigarette smoke represents a critical health problem (Surgeon General, 2002). Toxicological

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effects of perinatal cigarette smoke exposure include lower birth weight (Eskanazi et al., 1995), higher rate of spontaneous abortion (Kline et al., 1997), and increased incidence of sudden infant death syndrome (SIDS) (Bulterys, 1990). Some effects can have long-term consequences. For example, maternal smoking during pregnancy can lead to increased aggression (Weissman et al., 1999), and problems with sustained attention and impulsivity in adolescent offspring (Fried et al., 1992). Children of smoking mothers are at increased risk for attention-deficit/hyperactivity disorder (Milberger et al., 1997; Wasserman et al., 1999), conduct disorders (Fergusson et al., 1997; Wakschlag et al., 1997) and drug abuse (Weissman et al., 1999), and they are responsible for high rates of violent and persistent criminal offenses (Rasanen et al., 1999; Weissman et al., 1999).

Behavioral deficits generally related to arousal and attentional problems in humans have been identified in rats exposed to NIC prenatally. These animal models show deficits in attention and memory in maze performance (Levin et al., 1993; Sorenson et al., 1991), in learning (Levin et al., 1993), and in operant behaviors (Martin and Becker, 1971). Prenatal NIC exposure is associated with problems with neuronal growth and path-finding (Zheng, 1994), and cell proliferation and differentiation (Lajtha, 1985; Slotkin et al., 1987). Prenatal NIC exposure can induce up-regulation of nicotinic receptors (Sershen et al., 1982), to a greater extent on $\alpha 4\beta 2$ subunit containing receptors than on $\alpha 7$ subunit-containing receptors (Van de Kamp and Collins, 1994).

We have been investigating the effects of prenatal exposure to cigarette smoke on brain regions involved in arousal and attention. The pedunculopontine nucleus (PPN), part of the reticular activating system (RAS), is involved in the control of arousal, waking and rapid eye movement (REM) sleep, and disturbances during the development of REM sleep have been proposed to lead to a number of arousal, attentional and behavioral deficits later in life (Garcia-Rill et al., 2003). Figure 1 shows the major connections of the RAS, especially projections from the PPN to the intralaminar thalamus that are considered to modulate thalamocortical rhythms. We hypothesized that a lack of developmental decrement in REM sleep will lead to lifelong increases in REM sleep drive, such as are present in schizophrenia, anxiety disorders and depression (Garcia-Rill et al., 1997, 2003). Cells in the region of the PPN express $\alpha 4\beta 2$ subunit containing nicotinic receptors (Tribollet et al., 2004). Therefore, we developed a method for exposing pregnant dams to cigarette smoke and studying the effects of such exposure on a) the intrinsic membrane properties of PPN neurons during the developmental decrease in REM sleep, and b) the manifestation of the vertex-recorded P13 potential (the rodent equivalent of the P50 midlatency auditory evoked response) which is considered to reflect PPN output in the freely moving animal. These animal models provide an opportunity to characterize cigarette smoke exposure-induced toxicological changes in PPN activity as one of the potential mechanisms underlying the arousal and attentional deficits observed in the offspring of mothers who smoked during pregnancy. The results described herein suggest that major disturbances in the intrinsic membrane properties of PPN neurons, as well as in the manifestation of PPN-generated waveforms in freely moving adolescent animals, could lead to hypervigilance and attentional dysregulation later in life. Preliminary results have been reported in abstract form (Garcia-Rill and Buchanan, 2006).

Materials and Methods

Subjects

Pups born to adult timed pregnant Sprague-Dawley rats (Harlan 200–250g) were used. Pregnant dams were received at embryonic day 10 (E10) and were housed individually in a vivarium with 12:12 light/dark schedule (lights on at 0600 hr) and food and water *ad libitum*. Litters were culled to 10 animals before postnatal day 10. In some litters, when the

pups were 12–21 days old, they were anesthetized and rapidly decapitated as previously described (Kobayashi et al., 2003, 2004a, b, c). The brains were dissected free in cooled, oxygenated artificial cerebrospinal fluid (aCSF), and 400 μ m slices cut for intracellular recording as previously described (Kobayashi et al., 2003, 2004a, b, c). In other litters, when the pups were 28–30 days old, they were anesthetized and implanted for recording of the P13 potential as previously described (Homma et al., 2003; Mamiya et al., 2005). All animal use procedures were approved by the UAMS and ASU Institutional Animal Care and Use Committees and comply with the ethical standards described in the NIH Guide.

Smoke exposure

Since PPN neurons are born at E12–14 (Phelps et al., 1990), on day E11, pregnant dams were accommodated to a Plexiglas smoke exposure chamber, and on day 12 exposed once/day, on day 13 twice/day, and on day E14 until parturition, animals were exposed to 150 ml of cigarette smoke from a 1R3F experimental cigarette (Kentucky Tobacco Research and Development Center; 15 mg tar, 1.16 mg nicotine per cigarette) for 15 min 3 times/day. Smoke was generated by a CH Technologies, model 625A/SG-100 cigarette smoking machine and delivered to the exposure chamber. The amount of NIC in 350 ml of smoke from the 1R3F cigarettes was 204 ± 16 μ g (mean $\pm$ S.D., $n=4$). The maximum instantaneous concentration of NIC to which animals were exposed was 24 ± 1.9 ppm. The average exposure over the 15 min exposure period was 9.7 ± 0.8 ppm NIC.

The amount of carbon monoxide (CO) in the chamber was monitored every 2 min using a Monoxor II CO meter (Bacharach, Inc, New Kensington, PA). NIC was extracted using anhydrous 2-propanol (Sigma, St. Louis, MO) and stored over a 4 Å molecular sieve to ensure dryness. A Perkin-Elmer Clarus 500 GC-MS equipped with an autosampler and controlled by a PC running Perkin Elmer Turbo Mass 4.5 software was used for all analyses (Perkin-Elmer, Wellesley, MA). The separation of NIC and COT from other substances extracted from the plasma sample was performed using a Perkin-Elmer Elite 5MS column (25 m long, 0.20 mm ID, 0.33 mm film thickness). Calibration standards were from certified (–)-COT and S(–)-NIC solutions obtained from Cerilliant, Inc. (Round Rock, TX).

Recording Procedures

Brainstem slices (12–21 days)—The slices were suspended between nylon mesh in a chamber that allowed warmed oxygenated aCSF to flow around the slice and intracellular recordings carried out as previously described (Kobayashi et al., 2003, 2004a, b, c). The properties measured included membrane input resistance (R_{in}), determined by hyperpolarizing pulses 500 ms in duration at 0.1–0.3 nA applied at resting membrane potential (RMP)), action potential (AP) amplitude duration and threshold, and afterhyperpolarization (AHP) amplitude and duration. Changes in the hyperpolarization-activated cation I_h current were also measured across age in both control and treated animals. Neuroactive agents were applied via gravity-fed superfusion as follows: the selective I_h blocker ZD-7288 (50 μ M), and the sodium voltage gated channel blocker tetrodotoxin (TTX, 0.3 μ M). Measurements as described above were carried out before, during and after recovery from exposure to neuroactive agents. After recordings were completed, the neuron was iontophoretically injected with neurobiotin using intracellular depolarizing pulses of 500 ms duration at 1 Hz for 15 min, and adjusted to elicit a train of APs (around 0.5–1.0 nA). All of the slices were processed for NADPH diaphorase histochemistry to selectively label cholinergic mesopontine (PPN) neurons (dark blue or purple cytoplasm evident in light microscopy) and the location of each recorded cell among NADPH diaphorase-labeled neurons within the PPN was verified.

P13 potential (30–60 days)—Control untreated animals and offspring of dams exposed to cigarette smoke during pregnancy were allowed to age until 30 days, then implanted with recording electrodes as previously described (Homma et al., 2003; Mamiya et al., 2005). Auditory evoked potentials were recorded from the vertex (Vx) of unrestrained, alert rats placed in a sound-attenuating chamber. Stimulation consisted of pairs of 100 usec clicks delivered at a 500 msec interstimulus interval and a 5 sec intertrial interval until 32 pairs of evoked potentials had been averaged. Evoked potentials from the Vx were amplified (10,000x) and bandpass filtered at 3 Hz–1 kHz. Measurement of the amplitude of the P13 auditory evoked potential, a positive waveform starting at a latency of 7–9 msec and peaking at 12–14 msec (Miyazato et al, 1999a, 1999b, 1999c, 2000a), was made from the beginning of the wave to its peak, while habituation was calculated as the amplitude of the potential induced by the second stimulus as a percent of the amplitude of the potential induced by the first stimulus.

Statistical Procedures

Note that responses from all recorded cells in the pups of each litter were used to derive a mean for each litter, then a mean of the mean of the litters was derived from averaging the means from the 4 litters, whether intact or treated. For the P13 potential, we recorded from 4 pups in each litter and a mean for each litter calculated, then a mean of the mean of the litters was derived, whether intact or treated. To compare data between the different age groups, drug effects and cell types in the experiments, measures were tested using one-factor, two-factor or multifactor analysis of variance (ANOVA) in order to determine if any of the factors had a significant effect on the magnitude of the measure, and also whether the interaction of the factors significantly affected the measure. Differences were only considered significant at values of $P \leq 0.05$. If a statistical difference was found, a post-hoc test (Newman-Keuls) was used to compare between groups. Each of the measures of intrinsic membrane properties across age was compared using a two-factor ANOVA.

Results

Pregnant dams exposed to cigarette smoke showed no evidence of stress and, usually initially explored the chamber and sat quietly during smoke exposure. During the highest concentrations used they were not alert or responsive to activity outside the chamber. After smoke exposure, the animals again moved freely in the chamber. Timed-pregnant dams were used so that pregnancy rate was not an issue, and there were no spontaneously aborted pregnancies in treated rats. Exposed dams weighed 220 ± 27 gm at day E12 and 290 ± 43 gm before parturition, compared to unexposed dams (220 ± 23 gm vs 290 ± 21 gm). Exposure to cigarette smoke appeared to have had no effect on maternal weight gain during pregnancy. Fetal body weights of pups of exposed dams was 21 ± 1 gm, similar to those born to control dams (20 ± 1 gm). Brain weights were not taken due to the need for rapid dissection of brain tissue required to generate viable slices. Previous studies showed doses of cigarette smoke tolerated by the pregnant rats were similar to those used here did not induce differences in fetal weight, length, or other teratological measures (Reczeh et al., 1975). However, recent studies in the mouse have shown that tobacco smoke produces a dose-related retardation in embryonic growth (Seller and Bnait, 1995). NIC appeared to lead to maturational delays in cholinergic brain systems, but did not produce overt signs of maternal or fetal toxicity (Zahalka et al., 1992). In general, our results suggest that cigarette smoke is not a potent teratogen but is detrimental to the fetus.

Brainstem slices

Forty (40) pups were studied from 4 dams exposed to cigarette smoke from E12 until birth. Forty (40) pups from 4 unexposed dams were used. There are three types of PPN neurons

based on their electrophysiological characteristics (Kobayashi et al., 2003, 2004a, b, c). Type I cells have low threshold spike (LTS) conductances and are non-cholinergic, type II cells have *I_a* conductances (or A-current) and two thirds are cholinergic, and type III cells have both LTS and *I_a* conductances and one third are cholinergic. Only type II cells were studied in the present experiments. Previous studies showed that AP duration (measured at half the amplitude of the AP) decreased from (mean±SE) 2.1±0.9 ms to 1.3±0.5 ms (average 1.5±0.6 ms) between 12 and 21 days, but AP amplitude, AP threshold, RMP, Rin, AHP duration, and AHP amplitude did not change in PPN neurons during this period (Kobayashi et al., 2003, 2004a, b, c). Because litter effects can be large and obscure or bias treatment-related effects, we analyzed average membrane characteristics per litter. There were no differences across litters from control (n=4) and cigarette smoke-exposed (n=4) dams. Table I lists the mean±S.E. of the membrane properties measured, namely, RMP, AP threshold, AP amplitude, AP duration, AHP duration, AHP amplitude and Rin. The cells sampled were distributed across the litters in each group, allowing statistical comparisons within groups of litters. There were no statistical differences across litters within each group, control or cigarette smoke-exposed, suggesting that there were no litter effects in this study.

Table I shows the basic membrane properties of 32 cells recorded in intact animals from 4 dams compared to those of 27 cells from 4 dams exposed to cigarette smoke during pregnancy. The values from untreated animals were similar to those previously reported, i.e. AP duration 1.5±0.1 ms, AP amplitude 51±1 mV, AP threshold -57±1 mV, RMP -66±1 mV, Rin 107±6.3 MΩ, AHP duration 194±15 ms, and AHP amplitude 12±1 mV. On the other hand, some of the same intrinsic membrane properties of PPN neurons in cells from offspring of cigarette smoke-exposed dams were significantly different. RMP was decreased (-55±1 mV, ANOVA, df= 58, F=40.2, p<0.0001), AP threshold was decreased (-47±1 mV, ANOVA, df=58, F=41.9, p<0.0001), and AP duration was increased (2.5±0.2 ms, ANOVA, df=58, F=20.6, p<0.0001) in type II cells from the offspring of treated dams compared to those from untreated dams. AP amplitude, Rin, AHP duration and AHP amplitude were unchanged.

A feature in the majority of type II PPN neurons is the hyperpolarization-activated inward rectifying *I_h* current (Good et al., 2004). This current is carried by Na⁺ and K⁺, activates slowly, does not inactivate during prolonged hyperpolarization, and has a reversal potential (-30 to -50 mV) that is positive to RMP (Halliwell and Adams, 1982; Macaferri and McBain, 1996). *I_h* is thought to contribute to the RMP, rhythmic activity and other membrane properties in different regions (Macaferri and McBain, 1996; Lupica et al., 2001). Figure 2 shows measurements of the hyperpolarization-activated cation current *I_h* in cells from both offspring of control and of cigarette smoke-exposed dams. We reported previously that there was an increase in *I_h* amplitude during the developmental decrease in REM sleep (12–21 days) indicative of an increase in excitability during this period (Good et al., 2004). Therefore, in this study, cells were divided into two age groups, 12–16 and 17–21 days with 5 cells per group (note that not all cells had *I_h* so that the 5 cells in each group used were from 4 different litters), and *I_h* was measured as the difference from the peak membrane potential deflection elicited by 0.1–0.9 nA pulses to the level at which the hyperpolarizing pulse was released. *I_h* was activated only at certain membrane potentials (was not manifested at the lowest amplitude pulses), so that a cell was considered to have *I_h* if the membrane sag was seen in at least three of the nine current pulses that were delivered. *I_h* amplitude varied depending on the amplitude of the hyperpolarizing pulse, requiring a measure of varying amplitudes when *I_h* was manifested. Therefore, the 1/2 max amplitude was calculated by adding the minimum (excluding zero) and maximum amplitudes, and dividing by 2. The 1/2max amplitude of *I_h* in 12–16 day cells in control animals (1.6±0.2 mV, n=5 cells) compared to that in 17–21 day cells (5.1±0.2 mV, n=5 cells) was significantly higher later in development (ANOVA, df=9, F=134.4, p<0.0001), in agreement

with previous results (Good et al., 2004). *Ih* in cells from offspring of treated animals at 12–16 days (4.6 ± 0.7 mV, $n=5$ cells) was lower (ANOVA, $df=9$, $F=15.6$, $p<0.004$) compared to 17–21 day cells (9.9 ± 1.2 mV, $n=5$ cells). There was an age dependent increase in *Ih* amplitude in both cells from control animals and cells from treated animals. *Ih* amplitude in 12–16 day cells was significantly higher in cells from treated animals (ANOVA, $df=9$, $F=15.9$, $p<0.004$). *Ih* amplitude in 17–21 day cells was also significantly higher in cells from treated animals (ANOVA, $df=9$, $F=16.9$, $p<0.003$). This suggests that prenatal exposure to cigarette smoke increased further the amplitude of *Ih* throughout the developmental decrease in REM sleep.

The selective *Ih* blocker ZD-7288 was applied to type II PPN cells from offspring of cigarette smoke-exposed dams. The RMP prior to ZD-7288 was -54.0 ± 0.6 mV, whereas following ZD-7288 the RMP increased by 10 mV to -64.6 ± 2.9 mV, and was significantly higher (ANOVA, $df=9$, $F=13.0$, $p<0.007$). Cells from untreated animals following ZD-7288 had a mean RMP of -65.6 ± 2.3 mV, and the cells from treated animals following ZD-7288 had a similar mean RMP of -64.6 ± 2.9 mV (ANOVA, $df=9$, $F=0.07$, $p>0.8$). Following application of ZD-7288, the two main effects of ZD-7288 were that a) membrane potential increased to -65 mV, and b) *Ih* was blocked. These findings suggest that the significant increase in *Ih* amplitude in the cells from treated animals may account for the decrease in RMP seen in these cells. Some recordings were made while under TTX ($0.3 \mu\text{M}$) to prevent action potential firing (not shown). To ensure that this had no effect on *Ih* amplitude, we measured the amplitude of *Ih* before and after application of TTX and found no difference in *Ih* amplitude between these conditions (ANOVA $df=7$, $F=0.3$, NS).

P13 potential

Male pups from 8 litters, 4 born to exposed and 4 born to unexposed dams were prepared for recording evoked potentials between days 28 and 30. Note that only males are typically used for P13 potential studies in order to avoid fluctuations in sensory responsiveness across the menstrual cycle in females. Recordings of P13 evoked potentials began on day 35 and continued at 2–3 day intervals through day 60. A paired stimulus paradigm (interstimulus interval 500 ms) was used to investigate the effects of repetitive stimuli. The amplitude of the P13 response elicited by the 1st (conditioning) stimulus and the 2nd (test) stimulus was measured. The ratio between the amplitude of the test and conditioning stimuli was calculated for each trial (Figure 3).

To identify litter-specific differences, responses from all animals from each litter were pooled and compared using a one way ANOVA followed by Dunn's Multiple Comparisons test (DMC) to results from all other litters. This analysis revealed that there were no differences ($p>0.05$) between the 4 litters born to unexposed dams or the 4 litters born to exposed dams.

In general, the amplitude of the P13 potential to the conditioning stimulus of exposed litters was lower than that of unexposed litters (Figure 3A). However, comparison (one way ANOVA followed by DMC) of the responses of unexposed and exposed litters revealed that the amplitude of the P13 potential to the conditioning stimulus of only 1 exposed litter was significantly lower than that of the unexposed litters ($p<0.001$). Figure 3B shows that the amplitude of the P13 potential to the test stimulus of 3 of the exposed litters was higher than those of any of the unexposed litters. Statistical analysis (one way ANOVA followed by DMC) verified that the amplitude of the P13 potential to the test stimulus of 2 of the exposed litters was different ($p<0.01$) from the unexposed litters. Habituation of the P13 potential to repeated stimuli of the exposed litters was significantly lower ($p<0.01$) than that of all the unexposed litters (Figure 3C).

Discussion

These findings suggest that prenatal exposure to cigarette smoke leads to changes in the development of some of the intrinsic membrane properties of PPN neurons. The decreased RMP and AP threshold would tend to make neurons more excitable due to the changes in the driving force of cations, especially sodium. Such effects would increase tonic activity during waking, tending to increase arousal and synchronization of fast rhythms. The decrease in RMP and AP threshold may be due to a single cause, increased *I_h* amplitude. Channels mediating *I_h* are made up of four hyperpolarization-activated cation nucleotide-gated (HCN) subunits (1–4) (Biel et al., 1999; Ludwig et al., 1988; Santoro and Tibbs, 1999). The normal developmental increase in *I_h* amplitude in PPN neurons suggests a greater contribution of *I_h* to membrane excitability in more mature neurons (Good et al., 2004). The finding of even greater increases in *I_h* amplitude in PPN cells from offspring of cigarette smoke-exposed dams suggests that part of the decrease in RMP can be accounted for by increased *I_h* amplitude. Such a decrease in RMP will lead to a decrease in AP threshold and firing frequency, especially rhythmic firing during waking and REM sleep, would increase as a result. The increased amplitude of *I_h* also would lead to increase rhythmic firing in response to inhibitory inputs, since one role of *I_h* is to depolarize rhythmically in response to inhibitory inputs. This current, discovered in the heart, participates in oscillatory activity, which is important in various brain regions. All of these trends would increase tonic arousal levels during waking and REM sleep, since PPN neurons are known to be tonically active during waking and to increase bursting discharges during REM sleep (Steriade and McCarley, 2005). We speculate that HCN proteins may be affected by prenatal cigarette smoke exposure, and that such changes could be responsible for the arousal and attentional deficits (Milberger et al., 1997), as well as impulse control problems (exaggerated fight-vs-flight responses) reported in the children of mothers who smoked during pregnancy (Fried et al., 1992; Wasserman et al., 1999).

Pups that were exposed to NIC and the other constituents of cigarette smoke *in utero* had a small decrease in P13 potential amplitude following the 1st stimulus of the pair. This is similar to the effect of NIC injection on P13 potentials recorded from adults, which is considered a decrement in the level of arousal (Mamiya, et al, 2005). However, unlike adults exposed to NIC, pups exposed *in utero* to cigarette smoke also had an increased response to the 2nd stimulus. This suggests that maternal smoking also results in a decrement in the habituation of PPN responses to repetitive stimuli and is evidence that auditory gating in animals born to exposed dams is impaired. These *in vivo* results are consistent with the increased excitability of PPN neurons observed *in vitro*.

The effects of prenatal exposure to cigarette smoke thus appears to manifest itself in both a small decrement in level of arousal and a decrement in habituation to repetitive sensory input, an effect that persists until after puberty in the rat. The P13 potential has been found to undergo a decrement in amplitude following exposure to agents that decrease arousal level (e.g. alcohol, anesthetics, head trauma) (Miyazato et al., 1999c). Decreased habituation of the P13 potential has been observed after immobilization stress (Miyazato et al., 2000b). Evidence supporting the suggestion that the P13 potential is the rodent equivalent of the human P50 potential includes, a) the P13 potential, like the P50 potential, is sleep state-dependent, being present during waking and REM sleep and absent during slow wave sleep, b) there is a linear correlation between body weight of the species and the latency of this waveform (50 msec in human, 25 msec in feline, 13 msec in rodent), c) both are positive waves, d) both habituate rapidly, and both are blocked by the muscarinic antagonist scopolamine (Garcia-Rill and Skinner, 2001). The P50 potential has been found to manifest decreased amplitude in disorders that exhibit decrements in arousal, such as autism (Buchwald et al., 1992) and narcolepsy (Boop et al., 1994). Decrements in habituation of the

P50 potential are present in schizophrenia (Adler et al., 1982), anxiety disorders (Skinner et al., 1999), as well as autism (Buchwald et al., 1992) and other conditions (Garcia-Rill and Skinner, 2001). Therefore, our preliminary results on the effects of prenatal exposure to cigarette smoke on the pre- and post-pubertal manifestation of the P13 potential suggests the induction of a mild form of autism- or anxiety-like effects.

Implications of nicotinic input to PPN cells

Inhaled NIC is known to permeate the lungs where >80% of the available NIC is absorbed into the blood stream. The short delivery time and elimination half-lives (8 min and 2 hr, respectively) assure that, within a short time, the effect can be reproduced by smoking another cigarette (Benowitz, 1986). After absorption into the blood, NIC readily crosses the blood brain barrier and appears to be rapidly partitioned into brain tissue. For example, in rats, concentrations of NIC in the brain have been reported to be 5–7 times higher than blood concentrations (Gosheh et al., 2001). Although NIC is generally considered a stimulant, smokers assert that, in addition to its stimulatory effects on concentration and attention, the primary effect of smoking is that it calms and relaxes (Frith, 1972, Spielberger, 1986). Several investigators have reported that smoking increases during times of anxiety and stress (Conway et al., 1981, Pomerleau and Pomerleau, 1987). Some workers have verified these putative anxiolytic effects of NIC (Kassel and Shiffman, 1997, Kassel and Unrod, 2002), but others have not been able to establish such an effect (Fleming and Lombardo, 1987, Jarvik et al., 1989).

NIC has recently been reported to reduce hyperarousal and anxiety (Benowitz, 1996, Simosky et al., 2002). We recently found that the nicotinic agonist 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) (Phelan and Gallagher, 1992) depolarized PPN neurons early in development and later hyperpolarized them (Good et al., 2006). This effect was present during exposure to TTX and various receptor agonists, suggesting that at least some of the effects of DMPP were postsynaptic. However, the change during development (early excitation, later inhibition) mirrors the effects of the GABA_A agonist muscimol on PPN neurons (Bay et al., 2006). The possibility remains that DMPP may act through presynaptic excitation of GABAergic terminals that were not affected by TTX and the transmitter antagonists used (Good et al., 2006). Nevertheless, the important finding is that at least some PPN neurons were inhibited by this nicotinic agonist, at least initially. NIC may initially inhibit some PPN neurons to induce its calming effects, then activate additional brain regions, leading to the manifestation of its stimulatory effects. We recently reported that NIC, administered i.p., or the nicotinic agonist DMPP applied directly through cannulae into the PPN, worked to inhibit the P13 potential (the rat equivalent of the P50 potential that is sleep state-dependent and generated by the PPN) in a dose-dependent manner (Mamiya et al., 2005). This represents a novel action of NIC and possibly explains some of the anxiolytic effects that are initially seen after exposure to cigarette smoke, in keeping with the results with DMPP described here, i.e. net inhibition of at least some PPN neurons. This would be accomplished by an initial decrement in PPN output, i.e. decreased level of arousal. The present results suggest that some of this calming effect persists, but an additional decrement in habituation is also observed. Therefore, smoking may be a form of self-medication (Goff et al., 1992) intended to decrease the output of the RAS, i.e. the hypervigilance present in these disorders. We should note that this initial anxiolytic response may be followed by an anxiogenic one, since smoking has been reported to produce such effects (Benowitz, 1986, 1996; Fleming and Lombardo, 1987; Frith, 1972). We assume that such effects may be mediated by nicotinic activation of sites other than the PPN, since responses in PPN cells were hyperpolarizing after 16 days. If true, this push-pull (anxiolytic-anxiogenic) effect would explain the difficulty in shedding the smoking habit in the adult.

Conclusions

These preliminary results suggest that prenatal exposure to cigarette smoke produces marked increases in excitability in PPN neurons early in development, probably by increasing the amplitude of the hyperpolarization-activated cation current *I_h* conductance. The effects of such exposure persist into the pre- and post-pubertal period by mild decrements in the amplitude of the P13 potential, indicative of decreased arousal levels, as well as decrements in habituation to repetitive sensory inputs, indicative of a sensory gating deficit. These findings could explain some of the differences seen in individuals whose parents smoked during pregnancy, especially in terms of their hypervigilance and increased propensity for attentional deficits and cognitive/behavioral disorders.

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Figure 1. Ascending connections of the Reticular Activating System (RAS)

The dorsal cholinergic bundle (labeled PP in light yellow for PPN) from the RAS projects to the intralaminar thalamus (ILT) and serves to activate the cortex via thalamocortical projections (also in light yellow). Parallel monoaminergic (noradrenergic locus coeruleus (LC) projections in orange, and serotonergic raphe nuclei (RN) projections in blue) projections travel to the ILT and also directly to the cortex via the ventral bundle. These are joined by ascending projections from the TM (light green for histaminergic tuberomammillary nucleus). In turn, the TM sends descending projections to the RAS that may act reciprocally to stabilize sleep-wake states.

Figure 2. Change in *I_h* amplitude over the developmental decrease in REM sleep

A. Representative examples of type II PPN neurons with *I_h* currents. The amplitude of *I_h* in an individual record was measured from the peak of the hyperpolarization induced by the hyperpolarizing current step, to the membrane potential at which the current step was terminated. Note the lower amplitude of *I_h* in the 12 day and 20 day cells from offspring of an untreated dam (top row, CTL) compared to that in a 13 day and 18 day cell from offspring of a treated dam (second row, NIC), following application of the same amplitude hyperpolarizing pulses (0.5 nA). Calibration bar horizontal 100 ms, vertical 5 mV. **B.** Graph of the mean *I_h* amplitude in 12–16 day cells from offspring of untreated dams (filled triangles) (1.6 ± 0.2 mV) that increased significantly at 17–21 days (5.1 ± 0.2 mV). The mean

Ih amplitude of cells from offspring of treated dams (filled circles) also increased significantly between 12–16 days (4.6 ± 0.7 mV) and 17–21 days (9.9 ± 1.2 mV). *Ih* amplitude in cells from offspring of untreated dams (CTL) was significantly lower than that in cells from offspring of treated dams (NIC), regardless of age. **C.** Representative recordings from a 19 day type II PPN cell with *Ih* from an Exposed pup (left) and a 21 day type II PPN cell with *Ih* from an intact Control pup (right). Note the lower RMP (-54 mV) and greater amplitude of *Ih* in the cell from the Exposed animal compared to the higher RMP (-63 mV) and lower amplitude *Ih* in the Control pup. Calibration bar horizontal 150 ms, vertical 10 mV.

Figure 3. P13 potential findings

A. Representative examples of responses from individual Exposed and unexposed Control pups recorded on day 45. Waveforms shown are from 1 trial (average of 32 responses) recorded from each animal. The auditory click stimulus (99 dB) was given at the time shown by the vertical line to the left of each waveform. The 2nd stimulus was identical to the first but was given 500 ms later. Each panel shows the response elicited by a conditioning (left waveform) and test (right waveform) stimulus. The amplitude of the P13 response was measured from the beginning of the wave (~7–9 ms after stimulation) to its peak (~11–14 ms after stimulation). In both waveforms, the amplitude of the P13 response to the second or test stimulus (open circles) is smaller than the response to the first or conditioning stimulus (filled circles). The ratio between the responses to the test and conditioning stimuli (T/C

ratio) of the unexposed animal was 0.43 while the T/C ratio of the exposed animal was 0.80. In **B, C & D**, bars are the average ($M \pm SEM$) of 4 pups in each of 4 litters. In all figures, the shaded bars are $M \pm SEM$ of responses of 4 pups in each of the 4 exposed litters. **B.** Amplitudes of P13 responses elicited by a conditioning stimulus. The $M \pm SEM$ amplitude of only 4 pups from 1 of the exposed litters (asterisk) was different from that of the unexposed litters ($p < 0.001$). **C.** Amplitudes of the P13 responses elicited by a test stimulus. The average responses of 4 animals in each of 3 of the exposed litters were larger than those of the pups in the 4 unexposed litters. This difference was statistically significant for 2 of the litters (asterisks, $p < 0.01$). **D.** Habituation of the P13 responses of unexposed and exposed litters. Note that taller bars represent **reduced** habituation. Habituation was calculated as the ratio between the average amplitude of P13 response to the test stimulus and the conditioning stimulus for each trial. Habituation of P13 responses of all 4 of the exposed litters was significantly reduced compared to that of the 4 exposed litters ($p < 0.03$).

Table I

Electrophysiological properties of PPN neurons

Property	Control pups	Exposed pups	P value
RMP (mV)	-65.9±0.9	-55.0±1.4	<0.001
AP threshold (mV)	-56.5±0.7	-47.0±1.0	<0.001
AP amplitude (mV)	50.6±1.3	48.6±1.7	0.34
AP 1/2 amp. duration (ms)	1.5±0.1	2.5±0.2	<0.001
AHP amplitude (mV)	11.7±0.7	11.9±0.9	0.86
AHP duration (ms)	193±14	180±20	0.58
Input resistance (Mohm)	108±6	104±12	0.78
Ih 1/2 max. amplitude (mV)	3.3±0.3	9.9±1.1	<0.01

M±SE was calculated for all cells from pups in each of 4 litters and the mean of the mean for all 4 litters is listed below