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Prenatal nicotine alters maturation of breathing and neural circuits regulating respiratory control

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ABSTRACT

While perinatal nicotine effects on ventilation have been widely investigated, the prenatal impact of nicotine treatment during gestation on both breathing and neural circuits involved in respiratory control remains unknown. We examined the effects of nicotine, from embryonic day 5 (E5) to E20, on baseline ventilation, the two hypoxic ventilatory response components and *in vivo* tyrosine hydroxylase (TH) activity in carotid bodies and brainstem areas, assessed at postnatal day 7 (P7), P11 and P21. In pups prenatally exposed to nicotine, baseline ventilation and hypoxic ventilatory response were increased at P7 (+48%) and P11 (+46%), with increased tidal volume ($p < 0.05$). Hypoxia blunted frequency response at P7 and revealed unstable ventilation at P11. In carotid bodies, TH activity increased by 20% at P7 and decreased by 48% at P11 ($p < 0.05$). In most brainstem areas it was reduced by 20–33% until P11. Changes were resolved by P21. Prenatal nicotine led to postnatal ventilatory sequelae, partly resulting from impaired maturation of peripheral chemoreceptors and brainstem integrative sites.

1. Introduction

Maternal smoking during pregnancy induces a high incidence of ventilatory abnormalities in infants (apnea, delayed arousal responses, altered hypoxic ventilatory drive, or bronchopulmonary disease) with increased risk of Sudden Infant Death Syndrome (Mitchell and Milerad, 2006). Although the effects of prenatal nicotine on ventilation have been extensively studied in animals (Huang et al., 2004; Bamford et al., 1996; Bamford and Carroll, 1999; Simakajornboon et al., 2004), results remain conflicting, due to divergent experimental designs (species, nicotine administration route, time of pregnancy, duration and dose of nicotine administration, postnatal age) and types of ventilatory analysis (baseline data, apnea incidence, ventilatory response to hypoxia or hyperoxia). Most studies assessed changes in overall minute ventilation rather than in the two components: respiratory frequency and tidal volume. In addition, the cellular mechanisms underlying the control of ventilation after prenatal nicotine exposure remain partially elucidated.

The neural circuits regulating breathing belong to the chemoafferent pathway (Bianchi et al., 1995). In rats, the afferent chemosensory fibers arising from the carotid bodies project onto discrete areas of the medulla oblongata: mainly the caudal part of the nucleus tractus solitarius and, to a lesser extent, the ventrolateral medulla (Finley and Katz, 1992). The nucleus tractus solitarius and the ventrolateral medulla contain clusters of noradrenergic and adrenergic neurons: A2C2 and A1C1, respectively. The A2C2 cell group displays a functional subdivision: the caudal part (A2C2c) is influenced by peripheral chemosensory input and the rostral part (A2C2r) by barosensory input (Soulier et al., 1992). The A2 and A1 cell groups are involved in respiration through their connections to the adjacent dorsal and ventral respiratory groups. In addition, the A5 cell group, located in the ventrolateral pons and projecting onto the medulla oblongata and spinal cord, controls sympathetic output and respiratory events (Guyenet et al., 1993). The locus coeruleus (A6), the major noradrenergic cell group in the brain, belonging to the pontine tegmentum, with extensive descending projections onto the medulla oblongata and spinal cord, is involved in arousal

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and cardiorespiratory regulation and is part of the chemoreflex pathway (Guyenet et al., 1993; Hilaire et al., 2004). Catecholamines are the main neurotransmitters involved in chemoafferent pathway control. Firstly, dopamine is the most abundant neurotransmitter at the chemosensory synapse of the carotid body (Finley et al., 1992). Secondly, the central integration sites located in the nucleus tractus solitarius and the ventrolateral medulla are respectively associated with the A2C2 and the A1C1 catecholaminergic clusters (Finley and Katz, 1992). Thirdly, there is growing evidence that brainstem catecholaminergic neurons participate in the control of breathing. Mice invalidated for genes such as BDNF, mash, ret or rnx exhibited catecholaminergic impairment of carotid body, petrosal ganglion or brainstem noradrenergic system, systematically accompanied by ventilatory deficiency (Hilaire, 2006).

Maturation of these neural circuits develops within the first postnatal weeks and is dependent on the perinatal environment (White et al., 1994; Gauda et al., 2004; Donnelly, 2005; Wong-Riley and Liu, 2005). Prenatal nicotine reduces the brainstem noradrenaline level and/or utilization rate until weaning (Navarro et al., 1988), and induces cell damage, cell loss and synaptic dysfunction in the developing brain (Slotkin, 1998, 2004; Slotkin et al., 2007). It also enhances the expression of protein kinase C which plays an important role in both excitatory and inhibitory respiratory neurons (Bandla et al., 1999), thus possibly contributing to altered ventilation (Simakajornboon et al., 2004). In rat carotid bodies, perinatal exposure to nicotine upregulates tyrosine hydroxylase (TH) mRNA (Holgert et al., 1995; Gauda et al., 2001) and depresses breathing by attenuating the carotid body drive (Holgert et al., 1995).

Catecholamines are major components regulating ventilation at the peripheral and central levels (Finley and Katz, 1992; Bianchi et al., 1995), yet no studies have concomitantly investigated the effects of prenatal nicotine exposure on the development of breathing and catecholamine metabolism within the chemoafferent pathway. We therefore tested the hypothesis that prenatal nicotine exposure might cause abnormal breathing pattern development and that the impaired respiratory function might result in part from impaired development of the neural network regulating breathing.

In the present study, nicotine was delivered in pellet form throughout gestation from embryonic day 5 (E5) until birth; functional sequelae on resting ventilation and ventilatory response to acute hypoxic challenge were analyzed at postnatal days 7 (P7), 11 (P11) and 21 (P21). To determine whether nicotine-induced developmental abnormalities in ventilation were associated with changes in the postnatal maturation of ventilatory control, *in vivo* TH activity was analyzed at P7, P11 and P21 in the peripheral and central catecholaminergic structures of the chemoafferent pathway: *i.e.*, carotid bodies, A2C2 caudal (A2C2c) and rostral (A2C2r) part, A1C1, A5 and A6 brainstem cell groups.

2. Methods

2.1. Animals

Male and female Sprague–Dawley rats (IFFA Credo, France) were mated at night, and the morning on which sperm-positive smears were obtained was defined as embryonic day 0 (E0). Pregnant rats (300–320 g) were then housed in an air-conditioned room at $26 \pm 1\,^{\circ}C$ with a 12-h light–dark cycle and free access to food and water. They were randomly assigned to two groups: nicotine and control.

The nicotine group received the following treatment: pregnant dams were operated on at E5, so as not to disturb implantation of the embryo in the uterine wall. Dams were anesthetized with a single intraperitoneal injection of avertin (1 g tribromethanol dissolved in a mixture of 0.5 ml pentanol, 6 ml ethanol and 67.5 ml saline serum) (1 ml 100 g^{-1} body weight). Nicotine pellets (Interchim, Montluçon, France) were implanted subcutaneously between the scapulae. The nicotine group ($n = 45$) received a pellet delivering 50 mg free-base nicotine over 21 days, corresponding to a dose rate of 6 mg kg^{-1} day^{-1}. These dams gave birth to the group of "prenatal nicotine" pups.

The control group ($n = 30$), operated on at E5, received a placebo pellet (Interchim, Montluçon, France) delivering 50 mg vehicle for 21 days. These dams gave birth to the group of control pups.

At birth, prenatal nicotine pups were redistributed to nursing females never exposed to nicotine, to avoid any possible effects of nicotine and cotinine in the milk. Pups were randomized among all litters within each treatment group and litter sizes were randomly culled to 10 pups (8 males, 2 females) to ensure standard nutrition. Pups were housed with their mother and siblings until study. All animals were housed at $26 \pm 1\,^{\circ}C$ with a 12-h light–dark cycle. Only male rats were analyzed, to avoid gender differences in ventilation (Joseph et al., 2000).

Experiments were carried out according to the ethical principles laid down by the French (Ministère de l'Agriculture) and EU Council Directives for the care and use of laboratory animals (No. 02889).

2.2. Maternal plasma nicotine and cotinine measurement

To determine plasma nicotine and cotinine levels, 18 pregnant rats from the nicotine group were anesthetized (pentobarbital 0.15 ml 100 g^{-1} body weight intraperitoneally) at embryonic days E5 ($n = 3$), E8 ($n = 3$), E13 ($n = 3$), E16 ($n = 3$) and E21 ($n = 3$) and on postnatal day P5 ($n = 3$). Samples were taken by cardiac puncture. Blood was taken into a heparinized tube, light-protected and centrifuged. Plasma was frozen and stored at $-20\,^{\circ}C$ until assayed. Nicotine and cotinine levels were measured by HPLC-UV as previously described (Hariharan et al., 1988). The detection threshold was set at 0.012 nmol/ml for plasma nicotine and cotinine.

2.3. Ventilation measurement

Ventilation was measured in awake unrestrained male pups at P7, P11 and P21 using a barometric plethysmograph (Bartlett and Tenney, 1970). According to the protocol described previously (Peyronnet et al., 2000), the volume of the Plexiglas chambers was 0.22 l for P7 pups and 0.75 l for P11 and P21 pups. The Plexiglas plethysmographic chamber was connected to a reference chamber of the same size which was flushed with heated humidified air and both chambers were saturated with water vapor. Temperature and O_2 and CO_2 levels were continuously monitored. The system was calibrated by injecting a constant known volume of air with a syringe before placing an animal in the box, to avoid signal interference between calibration and respiration. Alternately, one control and one prenatal nicotine pups were weighed and placed in the chamber. Measurement began when the animal was calm. The gas flow was interrupted and the inlet and outlet tubes were closed. Breathing pressure fluctuations were recorded for 30–40 cycles with a differential pressure transducer (Celesco, CA, USA). The inspired CO_2 fraction was typically <0.5% at the end of the gas exposure period. Values for respiratory frequency (f_R, min^{-1}), tidal volume (V_T, ml 100 g^{-1}) and minute ventilation (V_E, ml min^{-1} 100 g^{-1}) were calculated breath-to-breath by computer analysis of the spirogram using standard methods. To assess the hypoxic ventilatory response (HVR), measurements were performed under baseline conditions (normoxia) and during hypoxic challenge (10% O_2 for 10 min).

Baseline measurements were made in quadruplets separated by 10 min intervals. The mean value was defined as the baseline