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Prenatal to Early Postnatal Nicotine Exposure Impairs Central Chemoreception and Modifies Breathing Pattern in Mouse Neonates: A Probable Link to Sudden Infant Death Syndrome

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Nicotine is a neuroteratogen and is the likely link between maternal cigarette smoking during pregnancy and sudden infant death syndrome (SIDS). Osmotic minipumps were implanted in 5–7 d CF1 pregnant mice to deliver nicotine bitartrate (60 mg Kg^{-1} day^{-1}) or saline (control) solutions for up to 28 d. Prenatal to early postnatal nicotine exposure did not modify the number of newborns per litter or their postnatal growth; however, nicotine-exposed neonates hypoventilated and had reduced responses to hypercarbia (inhalation of air enriched with 10% CO_2 for 20 min) and hypoxia (inhalation of 100% N_2 for 20 s) at postnatal days 0–3 (P0–P3). In contrast, at postnatal day 8, nicotine-exposed neonates were indistinguishable from controls. Isolated brainstem–spinal cord preparations obtained from P0 to P3 nicotine-exposed neonates showed fictive respiration with respiratory cycles longer and more irregular than those of controls, as indicated by high short- and long-term variability in Poincaré plots. In addition, their responses to acidification were reduced, indicating compromise of central chemoreception. Furthermore, the cholinergic contribution to central chemosensory responses switched from muscarinic receptor to nicotinic receptor-based mechanisms. No significant astrogliosis was detectable in the ventral respiratory group of neurons with glial fibrillary acidic protein immunohistochemistry. These results indicate that nicotine exposure affects the respiratory rhythm pattern generator and causes a decline in central chemoreception during early postnatal life. Consequently, breathing would become highly vulnerable, failing to respond to chemosensory demands. Such impairment could be related to the ventilatory abnormalities observed in SIDS.

Key words: **nicotine; central chemoreception; respiratory rhythm generator; sudden infant death syndrome; hypercarbia; hypoxia; acetylcholine**

Introduction

Maternal cigarette smoking increases the risk of sudden infant death syndrome (SIDS) (Mitchell et al., 1993; Kohlendorfer et al., 1998; Chong et al., 2004), a leading cause of death for infants <1 year old in developed countries (Dwyer and Ponsonby, 1995). Although the causes of SIDS are still unknown, most of the evidence points to failures in the generation of the respiratory rhythm or its modulation by chemosensory input (Nattie and Kinney, 2002). Infants who died from SIDS had previously shown alterations of their breathing pattern during sleep (Schechtman et al., 1991; Kahn et al., 1992), reduction of chemoreflexes (Shannon et al., 1977), central apnea (Kahn et al., 1988; Schechtman et al., 1991), and impairment in spontaneous and evoked arousability (Kahn et al., 2003).

Nicotine, a neuroteratogen present in tobacco, is the main candidate to be the factor linking cigarette smoking during pregnancy to cardiorespiratory dysfunctions (Slotkin, 1998; Nattie and Kinney, 2002; Hafström et al., 2005). Nicotine is transferred through the placenta into the fetus (Luck et al., 1985) where it can interact with functional nicotine receptors detectable from the early neural tube stage (Atluri et al., 2001). Postnatally, exposure to nicotine can be through breast milk or the environment (Luck and Nau, 1985). Although not all studies show similar results (Bamford et al., 1996; Slotkin et al., 1997; Bamford and Carroll, 1999), prenatal nicotine causes hypoventilation in rats (St-John and Leiter, 1999; Huang et al., 2004), increases the frequency of apnea during normoxia in mice and rats (Robinson et al., 2002;

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Huang et al., 2004), reduces hypoxia-induced ventilatory reflexes in awake rats and in sleeping lambs (St-John and Leiter, 1999; Hafström et al., 2002; Huang et al., 2004; Simakajornboon et al., 2004), reduces hypoxia-induced autoresuscitation from primary apnea in rat newborns (Fewell et al., 2001), and augments the delay in the awakening response induced by hypoxia in lambs (Hafström et al., 2002).

Anatomical and pathological studies of SIDS suggest involvement of brainstem chemosensory nuclei (Kinney et al., 2001; Nattie and Kinney, 2002). However, studies in animals have not consistently revealed that hypercarbia-induced ventilatory responses are compromised (Bamford and Carroll, 1999; Simakajornboon et al., 2004). Intense hypercarbia, which is expected during extreme physiological conditions, may be required to unmask subtle nicotine-induced deficits. Furthermore, little is known about the effects of prenatal nicotine exposure upon central chemoreception.

The aims of this work were to determine (1) whether prenatal to early postnatal nicotine exposure affects ventilatory responses evoked by severe hypercapnia and hypoxia in mouse neonates and (2) whether it affects fictive respiration and central chemoreception in the brainstem-spinal cord preparation in absence of peripheral sensory feedback. In addition, muscarinic and nicotinic receptor contributions to the central chemosensory responses were evaluated. Changes in glial fibrillary acidic protein (GFAP) immunostaining of neurons in the ventral respiratory group were studied to evaluate astroglial reactivity as a possible fingerprint of nicotine-mediated damage of respiratory related neurons.

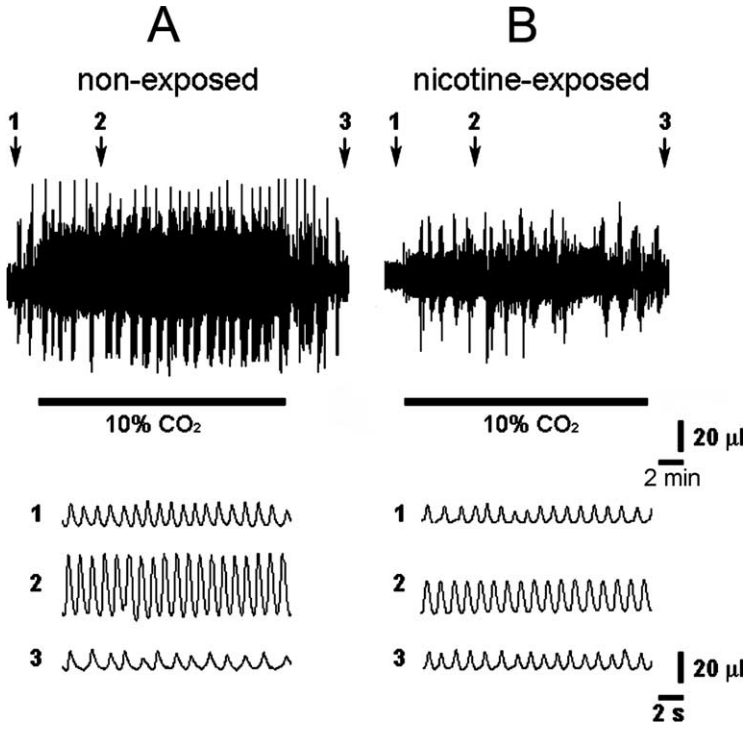

Figure 1. Ventilatory response to hypercarbia in control (***A***) and nicotine-exposed (***B***) P3 pups. Plethysmographic recordings of control (nonexposed) and nicotine-exposed P3 pups obtained before (arrow 1), during (arrow 2), and after (arrow 3) inhalation of air containing 10% CO_2. Fast sweeps of the recordings indicated by arrows are displayed in the respective traces below. Bar indicates period of hypercarbia. Note that large spikes correspond to oscillations produced by the displacement of trunk and extremities (movement artifacts). Measurements of ventilatory variables were done during periods that recordings were free of movement artifacts (traces 1–3).

Materials and Methods

Preparation. Experiments were done in accord with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and approved by the Bioethics committee of the Universidad de Santiago de Chile. At 5–7 d of gestation, and under strict aseptic conditions, 51 pregnant adult CF1 mice were anesthetized with ketamine/xylazine (80/20 mg Kg^{-1}, i.p.; Troy Laboratories; Alfasan International) and implanted subcutaneously with a 28 d osmotic minipump (2004, Alzet) through an incision made between scapulae. The pumps deliver saline (controls, $n = 27$) or nicotine bitartrate (60 mg Kg^{-1} day^{-1}, $n = 24$) at a rate of 0.25 $\mu l\ h^{-1}$. Osmotic minipumps were chosen to deliver nicotine for investigating mechanisms of fetal damage secondary to cigarette smoking, since it allows distinguishing between the effects of nicotine itself in the fetus from the effects of episodes of fetal hypoxia-ischemia produced by reduction of uterine blood flow. In addition, osmotic minipumps avoid the stress of daily injections of nicotine, as documented by increased sympathetic nervous system activity in dams (Suemaru et al., 1992; Houdi et al., 1995). Osmotic minipumps also mimic the tendency of smokers to maintain a constant blood level of nicotine throughout the day (Benowitz et al., 2002). In rats they keep steady-state plasma nicotine levels within the range (15–45 ng/ml) observed in the blood of pregnant women considered to be moderate smokers (Benowitz and Jacob, 1984) and in the amniotic fluid of smoke-exposed human fetuses (Luck et al., 1985). In fact, the physiological effects and steady-state blood levels obtained with osmotic minipumps are quite similar to those obtained by repeated daily injections of nicotine (Slotkin, 1998, 2004). In primates, both tobacco smoke exposure and nicotine infusion cause a similar upregulation of nicotinic acetylcholine receptors in the cortex and brainstem (Slotkin et al., 2002). This upregulation response has been used as a standard for nicotine's action. The dose regimen that we used in mice was 10 times higher than those used in rats, because 10-fold infusion rates are required in mice compared with rats to achieve a similar upregulation of (^{3}H)nicotine binding in the fetus (van de Kamp and Collins, 1994), particularly in respiratory-related regions of the brainstem (Pauly et al., 1991). This requirement seems to be related to differences in nicotine metabolism reflected in differences in plasmatic nicotine half-life: 5–7 min in mice (Petersen et al., 1984), compared with 54 min in rats (Plowchalk et al., 1992) and 2–2.5 h in humans (Benowitz and Jacob, 1993). In addition, Robinson et al. (2002) and we found that nicotine doses in mice were nontoxic, since they did not affect litter size or birth weight in mice. Indeed, higher doses of nicotine are needed to substantially decrease uterine blood flow in pregnant sheep (Monheit et al., 1983). Prophylactic antibiotic administration was not done; all dams recovered well without signs of infection. Dams were maintained in separate cages with water and food *ad libitum* at 22°C under a 12 h light/dark cycle. After finishing the experimental protocols, animals were killed with an anesthetic overdose.

Ventilatory recordings. Ventilatory recordings from awake P0, P1, P3, and P8 neonates were done using head-out body plethysmography. Neonates were placed in a 10 ml chamber sealed hermetically at the level of the neck with Parafilm, with their heads exposed. Inside the chamber the movements of neonates were restrained by fixing their hindlimbs. The temperature around the neonate body was maintained at 30°C by a temperature controller (FHC) which commanded an electric mantle in contact with the chamber while monitoring temperature with a thermistor (YSI, 44107) inserted through the chamber wall. The recording chamber was connected to one of the two ports of a differential pressure transducer (model DP103-14, Valydine Engineering), while the other was

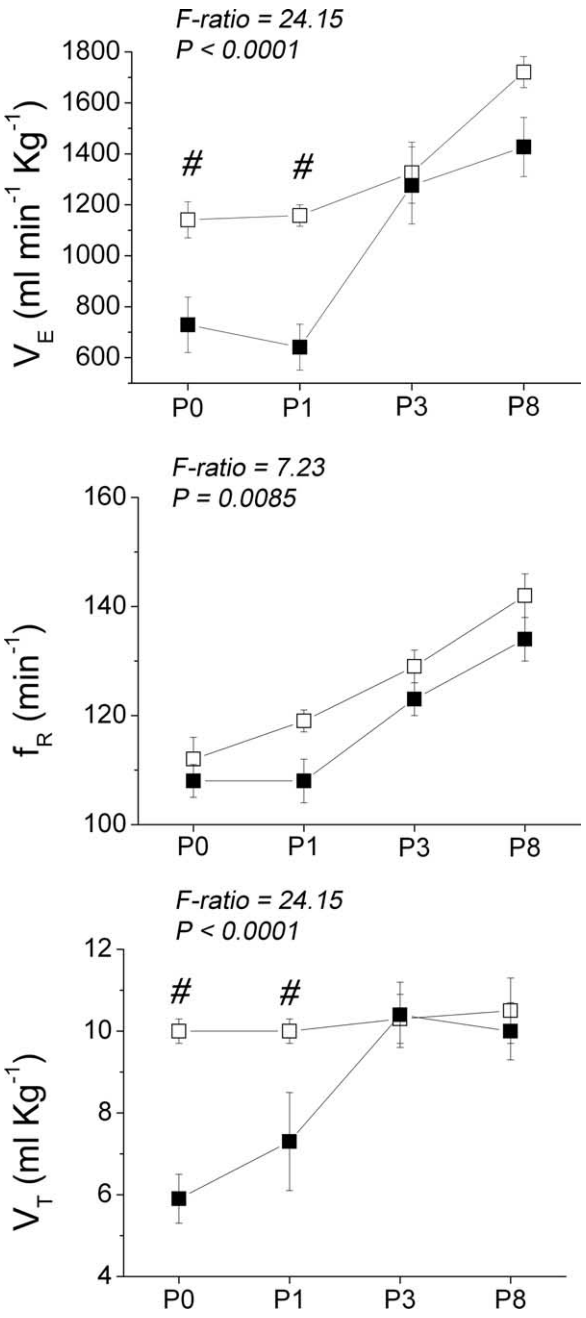

Figure 2. Mice exposed to prenatal-perinatal nicotine hypoventilate during early postnatal life. V_E, f_R, and V_T were evaluated in nicotine-exposed (filled squares, $n = 13$) and control (open squares, $n = 13$) pups. Data are expressed as mean ± SEM. ANOVA two-way analysis revealed a significant nicotine effect (df = 1, *F* ratios and respective *p* indicated for each variable) and a significant effect of aging upon V_E (*F* ratio = 14.63, df = 3, $p < 0.0001$), f_R (*F* ratio = 23.66, df = 3, $p < 0.0001$), and V_T (*F* ratio = 10.13, df = 3, $p < 0.0001$); $^{\#}p < 0.01$ indicates significant differences between nicotine-exposed pups and controls at a specific age as determined with the Newman–Keuls *post hoc* test. Note that V_E and V_T are normalized using the weights of the respective neonates.

connected to a reference chamber of identical volume. The signal was conditioned with a carrier demodulator (model CD 15, Valydine Engineering), and digitized at 1 kHz with an A/D acquisition system Digipack 1200B (Molecular Devices) connected to a computer controlled by Axoscope 9.0 software (Molecular Devices) for on-line oscilloscope mode display and posterior data analysis. Calibration was done by injecting volumes of 1, 5, 10, 20, and 50 μl through a small port into the recording chamber with a 10 or 50 μl Hamilton syringe.

Hypercarbic and hypoxic stimulation. Ventilatory recordings were done in 102 neonates (P0–P8). Evaluation of respiratory responses to hypercarbia or hypoxia began once neonates in the plethysmographic chamber were quiet and breathed regularly on air for at least 4 min. The head of the neonate was placed inside a hood through which gases were administered. The mixture of gases was humidified and their flow pressure maintained at 5 cm H_2O by passing it through the tip of a tube submerged 5 cm below the water surface in a bottle whose output was connected to the entrance of the hood. Administration of air through this system did not evoke any consistent change in amplitude or frequency of respiratory rhythm ($p = 0.78$, $n = 5$, Wilcoxon signed-ranks test) indicating that mechanical stimulation of the neonatal face with the puff of gasses did not interfere with our estimation of ventilatory responses to hypercarbia or hypoxia. A typical recording of hypercarbic stimulation consisted of an initial period of basal conditions in which neonates spontaneously breathed air during 2 min, then a period of stimulation in which neonates inhaled air enriched with 10% CO_2 (21% O_2 equilibrated in N_2) for 20 min, and finally a period of recovery in which neonates breathed air again for 8 min (Fig. 1*A*). Hypoxic stimulation consisted of an initial period of basal conditions breathing air for 2 min, followed by a 20 s period of hypoxia, in which neonates breathed 100% N_2, and finally an 8 min recovery period during which they again breathed air. More prolonged stimulation with 100% N2 was avoided, because it often resulted in the death of the neonate.

Evaluation of central chemoreception. Experiments were done in 76 newborns (P0–P3). Neonates were anesthetized with ether (Merck) and cooled on ice. As previously described (Infante et al., 2003), the CNS was removed and decerebrated through a ponto-mesencephalic transection, while it was immersed in artificial CSF (aCSF) containing (in mM): 125.0 NaCl, 5.0 KCl, 24.0–28.0 $NaHCO_3$, 1.25 $KH_2PO_4 \times H_2O$, 0.8 $CaCl_2$, 1.25 $MgSO_4 \times 7H_2O$ (Sigma), 30.0 D-glucose (Merck). The medium was equilibrated with O_2:CO_2 = 95%:5% (pH 7.40). The isolated brainstem/spinal-cord preparation was transferred to a recording chamber of 2 ml in volume. A thin film partition, sealed with Vaseline at C1–C2, allowed us to superfuse the brainstem separately from the spinal cord with a continuous flow of aCSF (0.8–2.0 ml min^{-1}).

Electrical recording. Spontaneous activity from C3–C5 ventral roots was recorded with glass suction electrodes at 24–25°C. Electrical signals were amplified by a low-noise differential amplifier (Almost Perfect Electronics), integrated with a full-wave rectifier (time constant = 100 ms), displayed on an oscilloscope (VC 6041, Hitachi), and recorded and analyzed with an Axoscope-Digipack 1320A AD acquisition system (Axon Instruments). The pH of the brainstem superfusion medium (7.1, 7.3, and 7.4) was obtained by equilibrating aCSF prepared with different final concentrations of sodium bicarbonate (12, 19, and 24 mM, respectively) with 95% O_2, 5% CO_2. Changes in sodium bicarbonate were compensated by reducing or increasing the concentration of NaCl to eliminate any osmotic effect. In previous work we showed that, in the range of concentration used here, changes in $[NaCl]_o$ did not alter fictive respiration (Eugenín et al., 2006). The tip of a micro-combination pH electrode (Model 9811, Orion) was placed into the recording chamber and connected to a pH/ion amplifier (Model 2000, A-M Systems) to record the pH of the superfusion medium. Before evaluation of the effects of acidification, ventral root activity had to be regular and stable for at least 3 min.

Evaluation of cholinergic contribution to changes in fictive respiration caused by acidification of the superfusion medium. Experiments were done in 70 newborns (P2–P3). Isolated "en bloc" brainstem–spinal cord preparations were placed in a two-compartment recording chamber with a Vaseline seal at C1–C2. As described above, fictive respiration was recorded from C4–C5 ventral roots while brainstem superfusion was switched from pH 7.4–7.1 in presence or absence of aCSF containing muscarinic acetylcholine receptor blocker, atropine 100 μM (Sigma), or nicotinic acetylcholine receptor blocker, hexamethonium chloride 100 μM (Sigma).

Immunohistochemistry. Neonates (P1–P3) were anesthetized by cooling and ether inhalation. Transcardiac perfusion with 10–20 ml of phosphate buffered saline (PBS: 137 mM NaCl; 10 mM Na_2HPO_4; 2.7 mM KCL; 1.8 mM KH_2PO_4; pH 7.4) was followed by 20 ml of fixative (4% paraformaldehyde and 0.1% picric acid in PBS). Brains were extracted, im-

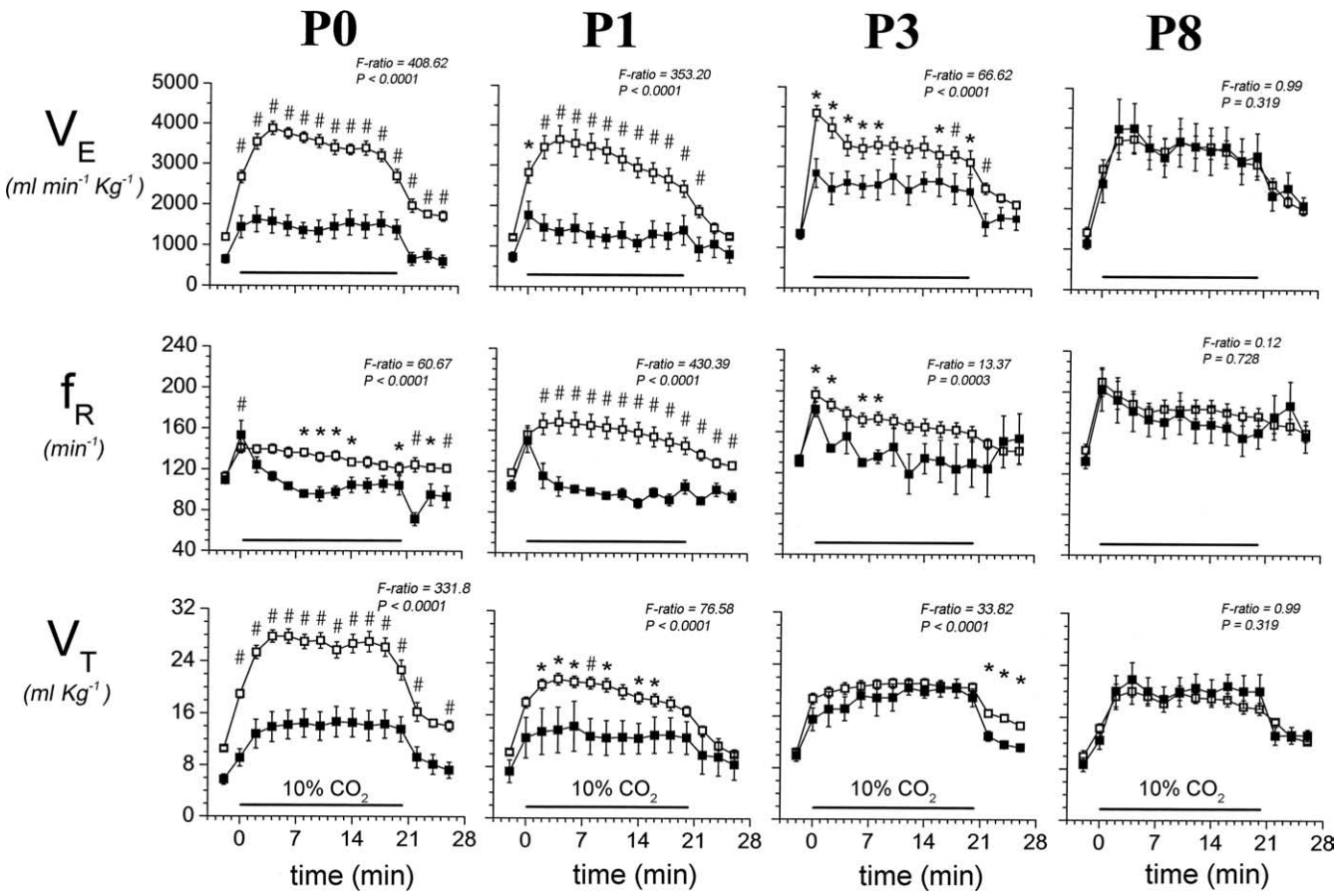

Figure 3. Prenatal-perinatal nicotine reduces the respiratory response to hypercarbia in early postnatal life in mouse neonates. Time course of changes in V_E, f_R, and V_T induced by hypercapnia (inhalation of air with 10% CO_2 for 20 min) in nicotine-exposed (filled squares, $n = 13$) and control (open squares, $n = 13$) pups. Hypercapnic stimulation is indicated by the bar. Data are expressed as means ± SEM. Significant nicotine effects upon V_E, f_R, and V_T were observed at P0, P1, and P3, but not in P8 (df = 1, *F* ratios and *p* indicated for each postnatal age). The symbols * and # indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) between nicotine-exposed pups and controls at a specific time, as determined with the Newman–Keuls *post hoc* test.

mersed in fixative for 24 h, transferred to 10% sucrose for 12 h, followed by 30% sucrose at 4°C for 24 h. Serial 10 μm thick transversal sections of the brainstem were obtained with a Leica cryostat (model CM1510), mounted on Superfrost* plus slides (VWR), and stored at −20°C. Recognition of the pre-Bötzinger complex was done by using polyclonal IgG goat anti-somatostatin (anti-SST) antibody (Santa Cruz Biotechnology). Astrocytes could be identified with polyclonal rabbit anti-GFAP (Dako) IgG. Sections were washed several times in PBS, incubated in bleaching solution (50 mM ammonium chloride in PBS) for 30 min, washed again 4 times for 5 min, and incubated in blocking solution (3% donkey serum, 0.2% Triton X-100, 0.01% thimerosal in PBS) for 1.5 h. Double staining (anti-SST + anti-GFAP) was obtained by incubation with primary antibodies diluted 1:500 in blocking solution at room temperature overnight. Then, sections were rinsed 4 times for 5 min with PBS, incubated with secondary antibodies, Alexa 546-donkey anti rabbit IgG and Alexa 488-donkey anti goat IgG (Invitrogen) 1:1000 in blocking solution supplemented with 1.5% donkey serum at room temperature for 2 h, and washed again with PBS. Sections were coated with Dako mounting medium and covered with glass coverslips. Observation of samples was done in a laser scanning system (LSM 510; Carl Zeiss) mounted on a motorized Axiovert 100-M microscope (Carl Zeiss) and equipped with a multiline argon laser (458 nm, 488 nm) and a helium-neon laser (543 nm). Images were acquired using a 40× objective, multitrack mode (505–550 nm BP, 585 nm LP) at 2 μm interval, and then averaged through LSM 510 software.

Quantification of glial cells in the ventral respiratory group was done by estimating the percentage of the GFAP-labeled area with respect to a total area of 230 × 230 μm captured as 512 × 512 pixel photographs using ImageJ (NHI) software. In brief, the 230 × 230 μm area was positioned on the ventral respiratory group containing SST-positive cells. The number of pixels in that region having a label intensity over a given threshold was determined, and expressed as percentage of the total in the region. The chosen threshold included all GFAP-positive cells, and excluded background. Measurements obtained from three different sections of a single animal were averaged at the level of the pre-Bötzinger complex (recognized by somata expressing somatostatin) and at a more caudal level containing somatostatin expressing terminals.

Measurement of nicotine in mice plasma. The determination of plasmatic nicotine concentration was performed by HPLC with UV detection (260 nm), according to Nakajima et al. (2000), with minor variations (Nakajima et al., 2000). Briefly, five dams were anesthetized with diethylether, decapitated, and exsanguinated 20 d after minipump insertion; the plasma sample (0.3–0.8 ml) was alkalinized with 50 μl of 12 N NaOH and then extracted with CH_2Cl_2 (1.5–3.0 ml) by shaking for 10 min. After centrifugation (5000 × *g* × 5 min at 8°C), the organic phase was separated and 10 μl of concentrated HCl was added. Solvent was evaporated under vacuum (40°C), the residue was dissolved in 70 μl of mobile phase (without solvents) and 50 μl of this sample were injected into the HPLC-UV system. The percentage of recovery was estimated by adding a known amount of nicotine to plasma samples of untreated animals (N = 7) and was determined to be 47.4 ± 5.3%. The chromatographic system consisted of a pump (model L6200A, Merck-Hitachi), a UV-visible detector (model L-4250, Merck-Hitachi) and a C18 column (Hypersil ODS 250 mm × 5 μm). The mobile phase flow was 1.8 ml/min and its composition was 164 mM citric acid (titrated with NaOH to a final pH of 3), 0.86 mM sodium octyl sulfate, and 4% tetrahydrofuran.

Data analysis. Newborn animals in each experimental group were ob-

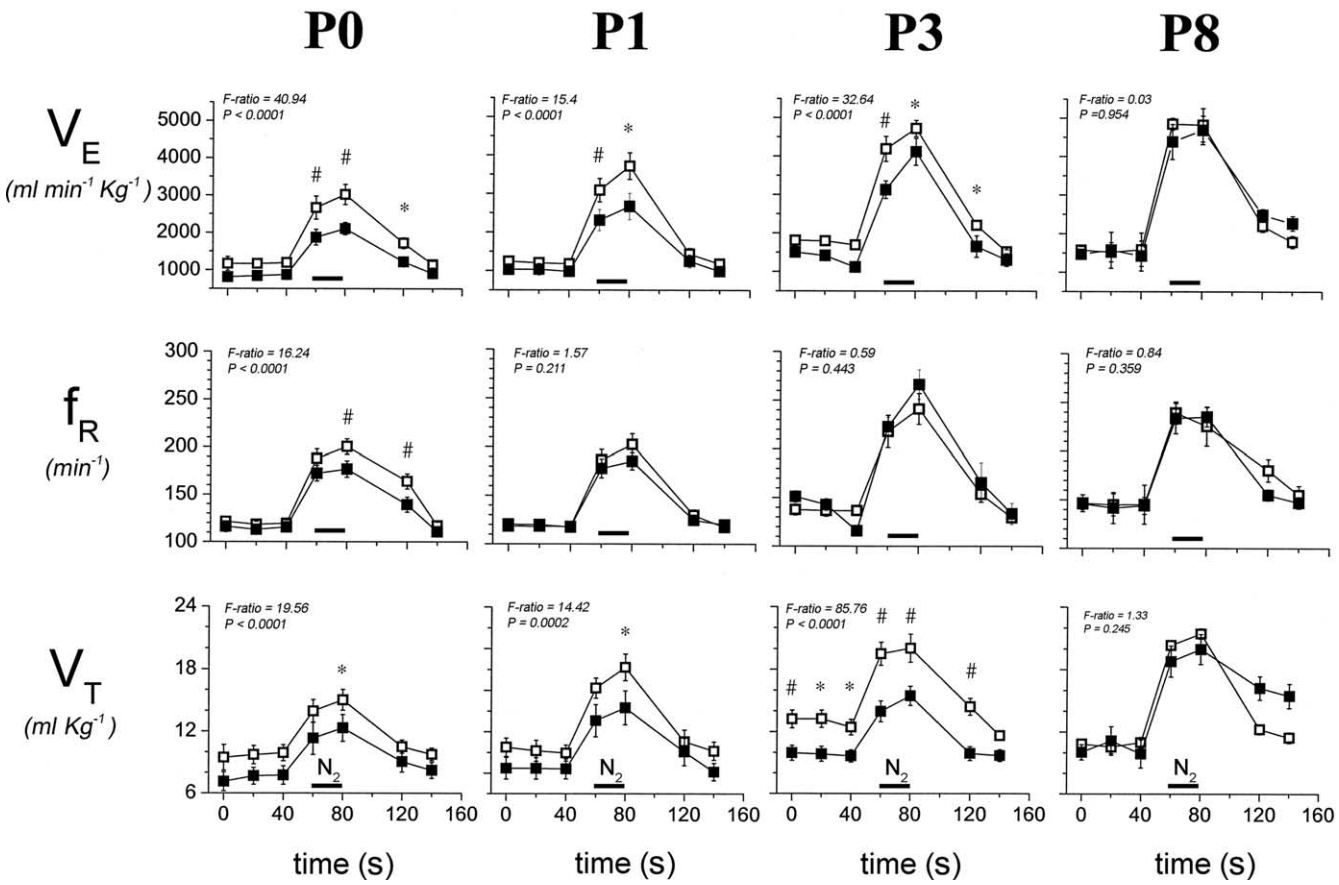

Figure 4. Prenatal-perinatal nicotine reduces the respiratory response to hypoxia in early postnatal life in mouse neonates. Time course of changes in V_E, f_R, and V_T induced by hypoxia (inhalation of 100% N2 for 20 s) in nicotine-exposed (filled squares, $n = 12$) and control (open squares, $n = 13$) pups; hypoxia administration is indicated by the bar. Data are expressed as means ± SEM. Significant effects of nicotine upon V_E and V_T were observed at P0, P1, and P3, but not P8 (df = 1, *F* ratios and *p* indicated for each postnatal age). Note that significant effects of nicotine upon f_R were observed only at P0; * and # indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) between nicotine-exposed pups and controls at a specific time as determined by the Newman–Keuls *post hoc* test.

tained from at least 3–4 litters. The number of pups per litter and their weights during P0, P1, P3 and P8, were noted for further analysis.

Tidal volume (V_T) and minute ventilation (V_E) were normalized by the respective weight of pups. V_T, expressed in ml Kg^{-1}, and instantaneous respiratory frequency (f_R), expressed in min^{-1}, were determined *in vivo* during the time course of the hypercarbic test at 2 min intervals from 20 consecutive cycles, whereas they were recorded at intervals of 20 s from 10 consecutive cycles during hypoxia. V_E, expressed in ml min^{-1} Kg^{-1}, was calculated multiplying tidal volume with the respective instantaneous respiratory frequency for each cycle. Apneas were defined as expiratory pauses equal or >3 s (Jacquin et al., 1996; Robinson et al., 2002) and their number and duration were measured before and after hypoxia challenge.

The amplitude of a burst of action potentials was estimated *in vitro* from the difference between the peak value of the integrated signal and the value of the integrated activity immediately before the onset of the burst, expressed in arbitrary units. Cycle duration was measured from the onset of one burst of action potentials to the onset of the next. Duration of the burst, which corresponded to the inspiratory duration for respiratory-like rhythm, was measured from the onset to offset of the burst. Instantaneous rhythm frequency was calculated from the reciprocal value of the cycle duration and expressed in min^{-1}. Values were expressed as mean ± SEM.

Differences between nicotine-exposed and control groups for the time courses of V_E, V_T, and f_R during hypercarbia or hypoxia were ascertained using a two-tailed *p* level estimated through a two-way ANOVA followed by Newman–Keuls test for *post hoc* pairwise comparison. Rejection of the null hypothesis was done if $p < 0.05$.

Cycle duration data were displayed in histograms and differences between distributions were assayed with Kolgomorov–Smirnov test. Poincaré plots were used to study the short-term and long-term rhythm generation variability. The Poincaré plot is a scatterplot of the duration of each cycle-to-cycle interval "T_n" on the *x*-axis plotted against the next cycle-to-cycle interval "T_{n+1}" on the *y*-axis. Thus, points above the line of identity indicate cycle-to-cycle intervals that are longer than the previous cycle-to-cycle interval, and points below the line of identity indicate a shorter cycle-to-cycle interval than the previous. In this analysis, the dispersion of points with respect to the line of identity is called the "width" and it is a measure of short-term variability, that is, cycle-to-cycle variability, while the dispersion along the line of identity, is called the "length" and it is a measure of long-term variability. The absolute value of the distance from each point along its orthogonal projection to the line of identity was used to quantify the width. However, the dispersion of the distances from points of orthogonal projections on the line of identity to the origin of the Cartesian plane was used to estimate the length. Comparisons of the width or length between control and nicotine-exposed preparations superfused at different values of pH were made using two-way ANOVA, followed by multiple comparisons with Newman–Keuls test for *post hoc* pairwise comparison.

Results

Litters and neonates

Prenatal nicotine exposure did not affect the litter size, which averaged 12.5 ± 1.5 newborns per litter in controls ($n = 23$) and 13.0 ± 2.0 in nicotine-exposed pups ($n = 20$; $p = 0.09$). It did not

affect the birth weight either, which reached 1.76 ± 0.12 g in controls ($n = 214$) and 1.73 ± 0.17 g in nicotine exposed ($n = 93$; $p = 0.40$) and neither affected the postnatal growth (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

Plasma nicotine

The maternal plasma nicotine concentration evaluated 4 weeks after implantation of the osmotic minipump was 207.1 ± 40.2 ng/ml (mean ± SEM; corrected for recovery; $n = 5$), which was in good agreement with that reported by Robinson et al. (2002) using similar nicotine doses and infusion rates.

Breathing pattern of P0–P8 neonates under eucapnic normoxic conditions is affected by prenatal to early postnatal nicotine exposure

Plethysmographic recordings showed periods of regular respiratory cycles, intermingled with occasional periods of either apnea or high amplitude and irregular oscillations produced by the displacement of trunk and extremities (Fig. 1, movement artifacts). In control pups, basal minute volume increased from P0 to P8 as a reflection of the postnatal increase in respiratory frequency, while the normalized tidal volume persisted ~10 ml Kg^{-1} (Fig. 2). The number of apnea periods decreased from 2.77 ± 0.86 min^{-1} in P0 ($n = 18$) to 0.36 ± 0.27 min^{-1} in P8 ($n = 11$, $p = 0.04$, ANOVA).

In nicotine-exposed pups, the postnatal evolution of the ventilatory variables was different from that observed in controls. Basal minute volume increased from P0 to P8. But in P0 and P1 it was lower than that observed in control pups (Figs. 1, 2). The reduction in basal minute volume was due, largely, to a pronounced reduction in basal tidal volume, and, to a lesser extent, to a decrease in basal respiratory frequency. The difference in basal minute ventilation between nicotine exposed and controls was not significant for either P3 or P8 pups, despite a reduction in basal respiratory frequency with age. As in controls, the number of apnea periods decreased with age, from 2.85 ± 0.35 min^{-1} in P0 ($n = 13$) to 0.89 ± 0.22 in P8 ($n = 9$, $p = 0.01$, ANOVA). No differences in the frequency and time spent in apnea were found between controls and nicotine-exposed mice (data not illustrated).

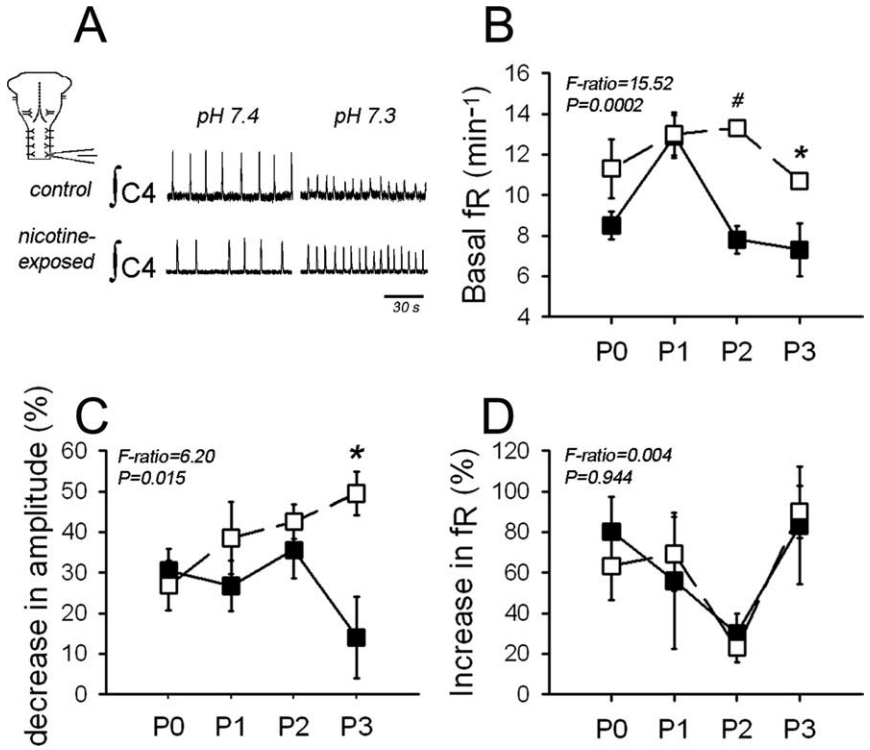

Figure 5. Prenatal-perinatal nicotine reduces both the frequency of fictive respiration and the response elicited by central chemoreceptor activation in the brainstem-spinal cord preparation from P0–P3 neonates. ***A***, Integrated inspiratory burst recorded from C4 ventral roots in isolated brainstem spinal cord preparations (see diagram in inset) obtained from control and nicotine-exposed P2 mice during superfusion with aCSF pH 7.4 and pH 7.3. Note the increase in frequency and the reduction in amplitude of the bursts induced by acidification. ***B***, Basal frequency of fictive respiration in nicotine-exposed preparations (filled squares, $n = 9$) is lower than those in controls (open squares, $n = 10$); reduction in amplitude and increase in frequency in response to acidification were expressed as percentage of the respective basal values. Changes in amplitude (***C***) were less pronounced, but no differences in frequency were observed (***D***) in nicotine-exposed animals compared with controls after changing the pH of the brainstem superfusion from pH 7.4 to 7.3. Data are expressed as mean ± SEM. ANOVA two-way analysis revealed a significant effect of nicotine upon basal frequency and response in amplitude induced by acidification (df = 1, *F* ratios and respective *p* indicated in each graph). Symbols * and # indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) at a specific age as determined by the Newman–Keuls *post hoc* test.

Effects of prenatal to early postnatal nicotine exposure upon ventilatory responses to hypercarbia

Inhalation of air enriched with 10% CO_2 increased the minute volume, the respiratory frequency and tidal volume in P0-P8 pups (Figs. 1, 3). The time course of the ventilatory responses induced by 20 min of hypercarbia is shown in Figure 3. The maximal values attained by respiratory frequency and minute volume during hypercarbia increased from P0 to P8. In contrast, the maximal value in normalized tidal volume induced by hypercarbia had a peak at P0, and then, from P1 to P8 it did not exceed 20 ml Kg^{-1}. In control pups, a typical response to hypercarbia consisted in a rapid and pronounced increase followed by either a plateau (P0–P1) or by a slight decline (P3–P8) in minute volume, which continued elevated above basal values while the stimulus was maintained. The time course profile for changes in respiratory frequency and tidal volume induced by hypercarbia in control pups, followed closely that of minute volume.

The ventilatory responses of P0, P1, and P3 nicotine-exposed pups were lower than those observed in the corresponding controls (Fig. 3). Values of minute and tidal volumes in nicotine-exposed pups persisted well below those observed in controls along the hypercarbic test. In contrast, the reduction of the response in respiratory frequency was evident only after the first 2 min of hypercarbic stimulation. Moreover, in P0 and P1 nicotine-exposed pups, respiratory frequency decreased during hypercarbia, dropping to values below basal levels. By P3, differences between the ventilatory responses of control and nicotine-exposed pups were still observed, but they were less pronounced. In contrast, by P8 the differences disappeared, and the time course of ventilatory responses of nicotine-exposed and control pups became indistinguishable. Only P0 and P1 nicotine-exposed pups regained basal values of minute volume during the first 8 min of recovery. In contrast, P0–P8 controls and P3–P8

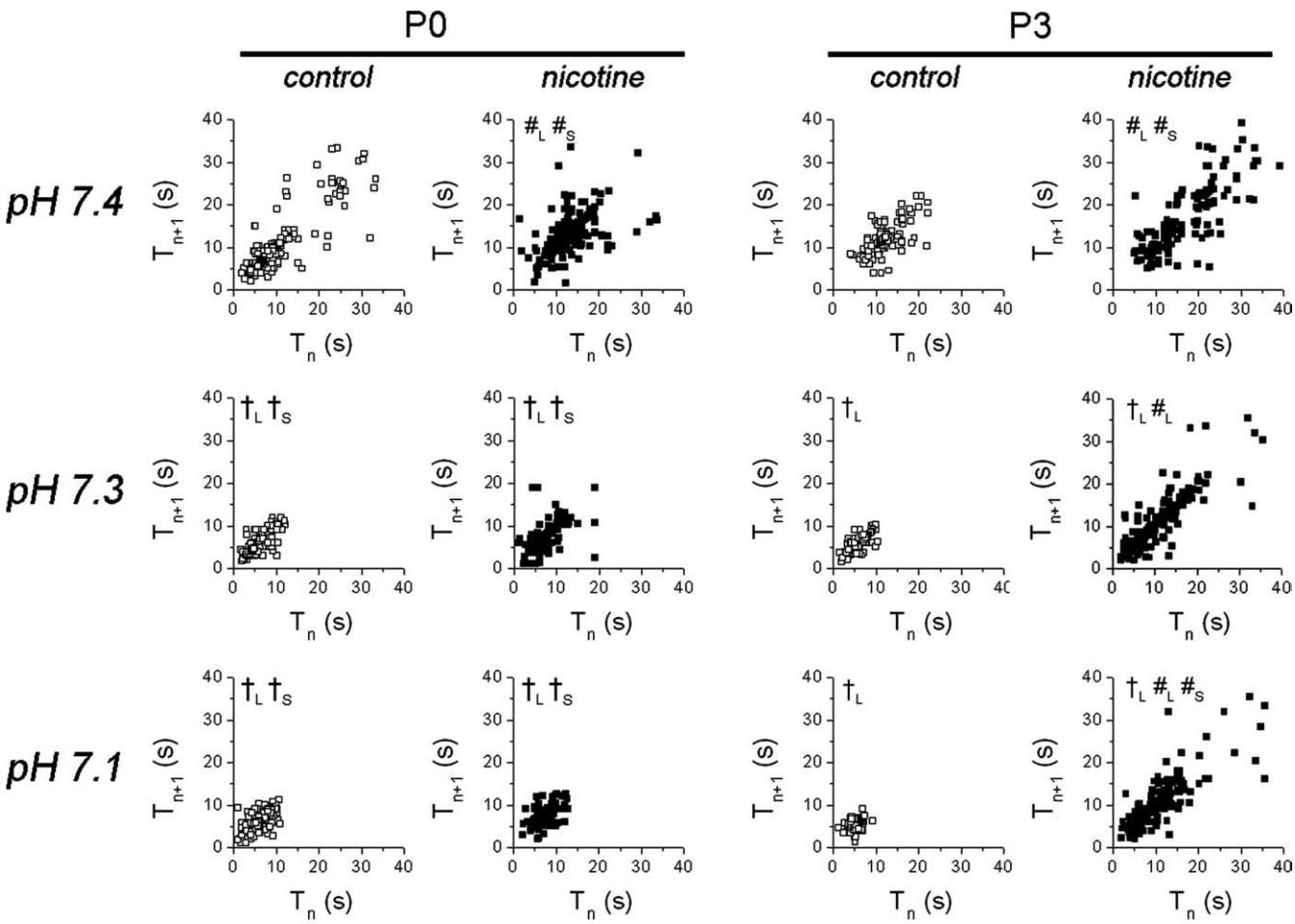

Figure 6. Poincaré plots of cycles T_{n+1} as function of the previous cycle, T_n, for fictive respiration recorded from brainstem–spinal cord preparations obtained from control (open squares, $n = 10$) and nicotine-exposed (filled squares, $n = 9$) P0 and P3 mice. Two-way ANOVA revealed a significant effect of nicotine upon long- and short-term variability in P0 (F ratio $= 119.45$, $p < 0.0001$; F ratio $= 13.16$, $p = 0.0003$, respectively) and P3 (F ratio $= 164.29$, $p < 0.0001$; F ratio $= 76.03$, $p < 0.0001$, respectively) pups. $^{\dagger}p < 0.01$, significant difference from pH 7.4. $^{\#}p < 0.01$, significant difference between nicotine-exposed and control preparations at specific pH conditions as determined with the Newman–Keuls *post hoc* test; subscripts L and S indicate long- and short-term variability, respectively.

nicotine- exposed pups required >8 min to recover their basal values (not illustrated).

Ventilatory responses to hypoxia after prenatal to early postnatal nicotine exposure

Inhalation of 100% N_2 increased respiratory frequency, tidal volume and minute volume (Fig. 4). The respiratory response to hypoxia consisted in a sudden increase in each ventilatory variable observed as early as the second or third cycle, followed by a slightly ascending ramp, peaking at the end of the 20 s stimulation (Fig. 4). The time course of the minute volume was similar to that of the respiratory frequency and tidal volume, all of which changed. Although less pronounced than hypercarbia, P0, P1, and P3, but not P8 nicotine-exposed pups had a reduced response to hypoxia (compare Figs. 3, 4).

Effects of prenatal to early postnatal nicotine exposure upon fictive respiration and central chemosensory drive

Brainstem–spinal cord preparations from both control and nicotine-exposed P0–P3 pups, showed spontaneous rhythmic activity from phrenic motoneurons. This fictive respiration consisted in bursts of action potentials appearing rhythmically at a frequency ranging from 3 to 18 min^{-1} (Fig. 5*A*). Preparations obtained from P0, P2, P3, but not P1 nicotine-exposed pups showed a lower basal frequency than that found in controls (Fig. 5*B*).

Both controls and nicotine-treated P0–P3 brainstem–spinal cord preparations showed a typical respiratory response to acidification (Infante et al., 2003; Eugenín et al., 2006). That is, switching the pH of the brainstem superfusion medium from 7.4–7.3 reduced the amplitude of the integrated inspiratory burst and increased the frequency of the fictive respiration (Fig. 5*A*). In both groups, the change in amplitude and frequency after acidification varied with postnatal age. While the reduction in amplitude was the least at P0 (P3>P2>P1>P0), the increase in frequency was the least at P2 (P0 similar to P3 and >P1>P2). Nicotine exposure decreased the change in amplitude of the integrated inspiratory burst activity (Fig. 5*C*) but did not modify the increase in frequency induced by acidification (Fig. 5*D*).

Effects of prenatal to early postnatal nicotine exposure upon respiratory rhythm *in vitro*

Cycle durations in nicotine-exposed preparations were longer and more dispersed than those observed in control preparations (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Differences between control and nicotine-exposed preparations were more pronounced at P2 and P3 than

P0 and P1. Acidification of the superfusion medium from pH 7.4–7.3 reduced cycle durations in both nicotine-exposed animals and controls, but differences between both groups persisted (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

Short-term and long-term cycle-to-cycle variability were studied using Poincaré plots. Comparison of Poincaré plots obtained from control preparations at pH 7.4 indicates that long-term variability is reduced from P0 to P3. Likewise, acidification of the superfusion medium from pH 7.4–7.3 or 7.1 in control preparations reduced short-term and long-term variability at the different postnatal ages. In contrast, P0 and P3 nicotine-exposed preparations showed higher short-term and long-term variability (Fig. 6). Moreover, a minimal or null reduction of variability induced by low pH was observed in preparations obtained from nicotine-exposed P3 pups. In fact, as illustrated in Figure 6, acidification did not reduce the cycle-to-cycle variability in P3 nicotine-exposed preparations.

Effects of prenatal to early postnatal nicotine exposure upon cholinergic contribution to brainstem respiratory chemoreception

To evaluate cholinergic contribution to central respiratory chemoreception, changes in fictive respiration induced by acidification of the superfusion medium (from pH 7.4–7.1) were recorded from brainstem-spinal cord preparations in the absence and presence of muscarinic (atropine 100 μM) and nicotinic (hexamethonium 100 μM) acetylcholine receptor blockers. Superfusion with atropine reduced the amplitude and frequency of basal, pH 7.4, fictive respiration by ~30% in both control and nicotine-exposed preparations ($p < 0.05$, ANOVA). In contrast, hexamethonium superfusion did not modify basal fictive respiration (data not illustrated).

Reductions in amplitude induced by acidification were statistically significant for controls in the absence of acetylcholine receptor blockers and in the presence of hexamethonium ($p < 0.01$, ANOVA), and for nicotine-exposed preparations in the absence of blockers and in presence of atropine ($p < 0.01$ ANOVA). However, increases in respiratory frequency induced by acidification were statistically significant for controls in the absence of acetylcholine receptor blockers ($p < 0.01$ ANOVA) and in the presence of hexamethonium ($p < 0.01$ ANOVA), and for nicotine-exposed preparations in absence of blockers and in presence of atropine. That is, in controls atropine but not hexamethonium suppressed the respiratory changes in response to acidification of the superfusion medium from pH 7.4–7.1 (Fig. 7). However, in nicotine-exposed preparations, atropine could not block the chemosensory responses. In contrast, hexamethonium, which does not modify the chemosensory responses in control preparations, abolished the expected increase in respiratory frequency induced by acidification (Fig. 7).

Absence of gliosis after prenatal to early postnatal exposure to nicotine

Because proliferation and accumulation of activated astrocytes occurs after neural tissue damage as part of the neuroinflammatory response, the presence of astrogliosis was assessed to evaluate possible damage secondary to nicotine exposure. Inspection of GFAP-positive cells in the ventral respiratory group in P1 and P3 mice, especially in the pre-Bötzinger complex as identified through somatostatin labeling, failed to show a significant increase in GFAP labeling after prenatal nicotine exposure. In contrast, there was a clear two-fold increase in GFAP labeling as the animals aged ($p = 0.04$, ANOVA), as expected for the normal development of astrocytes during early postnatal development (Fig. 8).

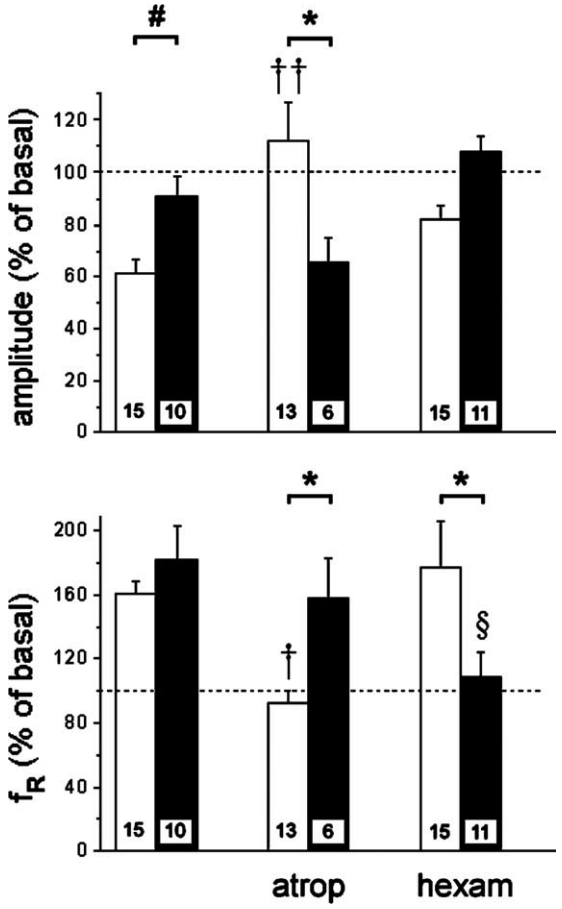

Figure 7. Cholinergic contribution to the central chemosensory responses is modified by prenatal to early postnatal nicotine exposure. Changes in amplitude (top graph) and frequency (bottom graph) of fictive respiration induced by acidification of the superfusion medium in isolated brainstem–spinal cord preparations are expressed as percentage of basal values. Control (open bars) and nicotine-exposed (filled bars) preparations from P2–P3 neonates were acidified in the absence or presence of muscarinic receptor blocker (atrop, atropine 100 μM) or nicotinic receptor blocker (hexam, hexamethonium 100 μM). ANOVA revealed significant differences in amplitude and frequency for control preparations (F ratio = 8.31, $p = 0.001$; F ratio = 5.14, $p = 0.01$, respectively). Note that muscarinic but not nicotinic blockade abolished the changes in amplitude and frequency ($^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ with respect to controls in the absence of blockers, Dunnett's test). In nicotine-exposed preparations, significant differences were observed in frequency, but not amplitude (F ratio = 4.14, $p = 0.03$; F ratio = 1.93, $p = 0.16$, respectively). In contrast to control preparations, nicotinic but not muscarinic blockade affected the acidification-induced responses ($^{\S}p < 0.05$ with respect to nicotine-exposed preparations in absence of blockers, Dunnett's test). Significant differences between control and nicotine-exposed preparations are indicated ($^{\#}p < 0.01$ and $^{*}p < 0.05$, ANOVA). Bars and vertical lines indicate mean and SEM, respectively, for data points. Numbers of preparations are indicated inside the bars. Dotted lines indicate no change with respect to basal values (100% of basal).

Discussion

Exogenous administration of acetylcholine agonists, like nicotine, may affect the normal development of the nervous system (Slotkin, 2004). Our results show that P0-P3 mouse neonates exposed to nicotine from E5-E7 fetal stages hypoventilate and have a reduction in the chemosensory responses to hypercarbia. This is the first direct evidence that prenatal to early postnatal nicotine disturbs central respiratory chemoreception, since it af-

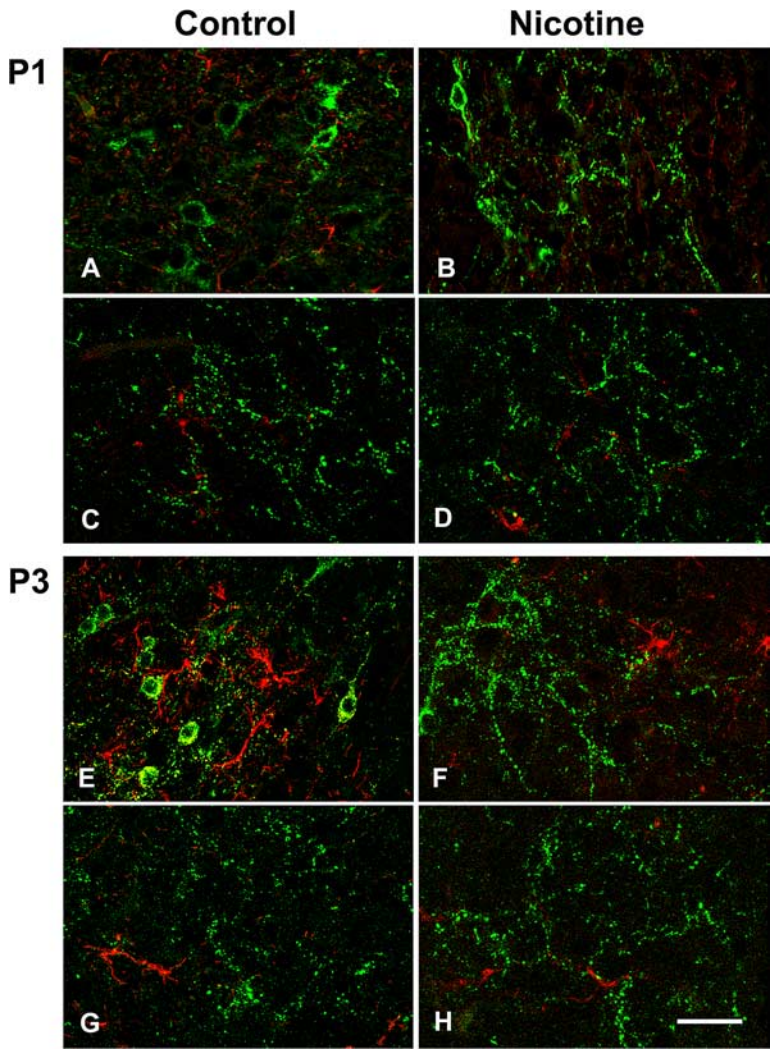

Figure 8. GFAP immunohistochemistry detected no gliosis in the respiratory ventral group region of P1 and P3 neonates after prenatal nicotine administration. In ***A***, ***B*** and ***E***, ***F***, somatostatin-positive cell bodies (in green) and GFAP-positive cells (astrocyte marker, in red) at the pre-Bötzinger complex of control and nicotine-exposed P1 and P3 neonates. In ***C***, ***D*** and ***G***, ***H***, somatostatin nerve endings in the ventral respiratory group (in green) and GFAP-positive cells (in red) of control and nicotine-exposed P1 and P3 neonates. Scale bar, 50 μm. Area occupied by GFAP-positive pixels was estimated from an area of 230 $\times$ 230 μm positioned on the ventral respiratory group using ImageJ software. GFAP labeling did not differ between controls ($n = 5$) and nicotine-exposed ($n = 6$) neonates evaluated in P1 and P3. However, it increased twofold from 1.2 $\pm$ 0.2% ($n = 10$) in P1 to 2.4 $\pm$ 0.4% ($n = 12$) in P3 neonates ($p = 0.04$, ANOVA).

fected the respiratory responses induced by acidification in the en bloc brainstem-spinal cord preparation in the absence of any peripheral input. Therefore, hypoventilation and reduced responses to hypercarbia in nicotine-exposed pups would be a major consequence of a deficit in the central chemosensory drive. In fact, we observed that, under basal conditions, pH 7.4, P0 and P3 nicotine-exposed preparations have a lower frequency and a higher cycle-to-cycle variability than those found in controls. Furthermore, in P3, but not in P0 nicotine-exposed preparations, acidification of the superfusion medium from pH 7.4 to 7.3 to 7.1 was unable to reduce cycle-to-cycle variability as seen in Poincaré plots. In contrast, in control mice the increased chemosensory drive at lower pH increases the frequency of fictive respiration and decreases the short-term and long-term cycle-to-cycle variability. It remains an open question whether the respiratory neural network has a minor response to the chemosensory input or the magnitude of this input provided by central chemoreceptors has decayed as a consequence of nicotine.

Our results indicate that a possible neural substrate underlying the respiratory deficit induced by prenatal nicotine can be central chemosensory nuclei. In the present study, we showed that the muscarinic cholinergic contribution to the central respiratory chemosensitivity (Monteau et al., 1990; Eugenín and Nicholls, 1997) was reduced and partially replaced by a nicotinic cholinergic contribution after prenatal nicotine exposure. To identify the particular subtypes of muscarinic and of nicotinic receptors that were altered by prenatal nicotine exposure would require pharmacological tools in addition to atropine and hexamethonium, used in this study**.** Prenatal nicotine exposure can reduce muscarinic receptor actions through uncoupling of G-protein dependent mechanisms, as described in rat striatum and hippocampus (Zahalka et al., 1993), or by reducing the binding of M2 muscarinic receptors in the rat brainstem at an early postnatal period (Slotkin et al., 1999), or by reducing mRNA of the muscarinic receptor in basal ganglia (Frank et al., 2001). However, it can increase nicotinic actions by receptor upregulation as described in the brainstem and cerebellum of rats (Slotkin et al., 2004). Interestingly, histopathology studies suggest that SIDS is related to a decrease in muscarinic receptor binding in the arcuate nucleus (Kinney et al., 1995), which would play an analogous role to ventral medullary surface of animals in contributing to the ventilatory and blood pressure responses to hypercarbia and asphyxia.

Our results, using a short and intense hypoxic test, suggest that peripheral arterial chemoreceptor input, the main sensory input regulating ventilatory chemoreflexes induced by hypoxia, is also affected by prenatal nicotine. This is in agreement with previous reports obtained in early postnatal rats and lambs, in which the ventilatory response to a "pure" hypoxic (not combined with hypercarbia) stimulus (St-John and Leiter, 1999; Hafström et al., 2002; Simakajornboon et al., 2004) or the "Dejours test" (inhalation of 100% O_2) (Bamford and Carroll, 1999) were measured. Cells belonging to the peripheral chemosensory input undergo modifications such as increased expression of mRNA for tyrosine hydroxylase in carotid body and petrosal ganglion in P0–P3 rats, and the augmented levels of dopamine β-hydroxylase in carotid bodies of P15 rats (Gauda et al., 2001). However, such nicotine-induced changes in peripheral chemoreceptor cells of 3- to 20-d-old rats do not appear to be associated with functional changes in carotid sinus nerve responses to hypoxia and hypercarbia (Bamford and Carroll, 1999). This could mean that a modification of the central integration of peripheral chemoreceptor input may play a major role in changes of the hypoxic chemoreflexes. In that sense, prenatal nicotine-induced changes in the GABAergic input into brainstem neurons involved in cardiorespiratory integration may

be an important mechanism influencing the gain of cardiorespiratory responses to hypoxia and hypercarbia (Neff et al., 2004; Huang et al., 2006; Luo et al., 2007).

However, other authors show no effect of prenatal nicotine exposure upon ventilatory hypoxic responses in neonatal rats (Bamford et al., 1996; Slotkin et al., 1997; Bamford and Carroll, 1999), or they report that prenatal nicotine affects the frequency and duration of apneas, but no other variable during hypoxic stimulation in P3 mouse neonates (Robinson et al., 2002). As discussed by Hafström et al. (2005), conflicting results can be attributed to differences in experimental protocols involving the use of anesthesia (Slotkin et al., 1997), the effects of central adaptation on hypoxic responses during the steady-state late response (Bamford et al., 1996; Bamford and Carroll, 1999), and the use of combined hypoxic hypercarbic gas mixtures (Bamford and Carroll, 1999), for example. That our results were different from those of Robinson et al. (2002) may seem, at first glance, puzzling, since both groups used the same species, with similar means of administration and dose of nicotine, and evaluated the early and the late phases of ventilatory response to hypoxia. However, our approach differed in the time of action of nicotine. In our experiments, fetal mice were exposed to nicotine from E5–E7 to postnatal days, a period more prolonged than the treatment from E10 to the end of pregnancy used by Robinson et al. (2002). This extended period allows nicotine to act during early events of embryogenesis, in particular, the whole segmentation period of the mouse hindbrain during formation of the rhombomeres (E8–E12) and, also, during postnatal days. In fact, depending on the developmental stage, exogenous nicotine administration can elicit a wide variety of responses (Slotkin et al., 2007). Furthermore, our use of a shorter and more intense hypoxic stimulation than that used by Robinson et al. (2002) might account for the different results.

In agreement with previous reports (Bamford and Carroll, 1999; St-John and Leiter, 1999; Hafström et al., 2002; Robinson et al., 2002; Simakajornboon et al., 2004), the dysfunction induced by perinatal nicotine upon ventilatory responses is transient and defines a vulnerable period from postnatal days 0 to 3–4. Whether this transient effect reflects a delayed process of maturation of the respiratory network or is the result of the time required for an adaptive reconfiguration of the network is unknown. Immunohistochemical evidence of gliosis, as expected following cell damage, was not detected in the region of the ventral respiratory group. Neuronal damage secondary to various mechanisms induces a 3- to 16-fold increase in GFAP label in neonatal animals (Khan et al., 2006; Svedin et al., 2007). Our results therefore indicate that neuronal damage is not a major mechanism in the pathogenic pathway linking nicotine to central respiratory dysfunction. They also suggest that brainstem gliosis observed in SIDS patients (Storm et al., 1999; Sawaguchi et al., 2003), showing threefold or even higher increase on GFAP labeling, might not be related directly to a cytotoxic effect of nicotine upon the respiratory network, but rather to the presence of other factors like chronic hypoxic-ischemic events.

In summary, this work supports the idea that prenatal to early postnatal nicotine exposure can transiently affect the respiratory pattern generation, causing a decline in central chemoreception and likely perturbing the central integration of peripheral chemoreflexes early in postnatal life. Consequently, breathing would be highly vulnerable, failing to respond to chemosensory demands. This could lead to the ventilatory abnormalities observed in diseases like sudden infant death syndrome and other neonatal respiratory pathologies associated to the consumption of tobacco.

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