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Alterations in cholinergic sensitivity of respiratory neurons induced by pre-natal nicotine: a mechanism for respiratory dysfunction in neonatal mice

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Nicotine may link cigarette smoking during pregnancy with sudden infant death syndrome (SIDS). Pre-natal nicotine leads to diminished ventilatory responses to hypercarbia and reduced central chemoreception in mice at post-natal days 0–3. We studied how pre-natal nicotine exposure changes the cholinergic contribution to central respiratory chemoreception in neonatal isolated brainstem–spinal cord and slice preparations.

Osmotic minipumps, implanted subcutaneously into 5–7 days pregnant mice, delivered saline or nicotine ditartrate 60 mg kg^{-1} d^{-1} for up to 28 days. In control preparations, acidification of the superfusion medium from pH 7.4 to 7.3 increased the frequency and reduced the amplitude of fictive respiration. In nicotine-exposed neonatal mice, the reduction in amplitude induced by acidification was reduced. In control preparations, atropine suppressed respiratory responses to acidification, while hexamethonium did not. By contrast, in nicotine-exposed preparations, hexamethonium blocked chemosensory responses but atropine did not.

Our results indicate that pre-natal nicotine exposure switches cholinergic mechanisms of central chemosensory responses from muscarinic receptors to nicotinic receptors. Modification of the cholinergic contribution to central chemoreception may produce respiratory dysfunctions, as suggested by receptor-binding studies in victims of SIDS.

Keywords: central chemoreception; respiratory rhythm generator; sudden infant death syndrome; hypercarbia; muscarinic receptors; nicotinic receptors

1. INTRODUCTION

Cholinergic inputs provide excitatory drive to neurons of the respiratory pattern generator (Weinstock 1981; Murakoshi *et al.* 1985; Gillis *et al.* 1988; Nattie & Li 1990; Burton *et al.* 1994, 1995; Shao & Feldman 2000, 2001, 2005; Hatori *et al.* 2006) and play a part in central chemosensitivity to H^+ and PCO_2 (Dev & Loeschcke 1979*a*,*b*; Fukuda & Loeschcke 1979; Nattie *et al.* 1989; Monteau *et al.* 1990; Burton *et al.* 1997; Eugenin & Nicholls 1997). The sites of acetylcholine action overlap with CO_2-chemosensitive regions, and the responses elicited by acetylcholine are similar to those elicited by low pH stimulation (Issa & Remmers 1992; Eugenin & Nicholls 1997). In addition, blockade of muscarinic receptors, which are expressed in various brainstem respiratory regions (Kinney *et al.* 1995*a*,*b*; Mallios *et al.* 1995), reduces respiratory responses to acidosis (Dev & Loeschcke 1979*a*,*b*; Monteau *et al.* 1990; Eugenin & Nicholls 1997).

If acetylcholine plays a neurotrophic role during brain development (Lauder & Schambra 1999; Gu 2002), treatment of a foetus with nicotine (a teratogen as well as an acetylcholine agonist) might give rise to changes in the properties of brainstem chemoreceptors in the neonatal animal (Mitchell *et al.* 1993; Kohlendorfer *et al.* 1998; Chong *et al.* 2004; Slotkin 2004). Indeed, there is evidence that links sudden infant death syndrome (SIDS), a cause of death in infants under 1 year old in developed countries, with smoking during pregnancy (Dwyer & Ponsonby 1995; Slotkin 1998). SIDS in turn could be due to abnormalities in the generation of the respiratory rhythm or its modulation by chemosensory input (Nattie & Kinney 2002; Eugenin *et al.* 2008). In fact, infants who died from SIDS had previously shown alterations of their breathing patterns during sleep (Schechtman *et al.* 1991; Kahn *et al.* 1992). Moreover, infant victims of SIDS or infants born of mothers who smoked showed a high incidence of central respiratory dysfunctions (Brady & McCann 1985), a major number of central apnoeas (Gennser *et al.* 1975; Kahn *et al.* 1988; Schechtman *et al.* 1991), diminished chemoreflexes (Shannon *et al.* 1977; Ueda *et al.* 1999) and decreased spontaneous and evoked arousability (Newman *et al.*

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1989; Schechtman *et al.* 1992; Lewis & Bosque 1995; Tirosh *et al.* 1996; Kahn *et al.* 2002, 2003; Horne *et al.* 2004). Anatomical and pathological studies of children with SIDS suggest that there are alterations in brainstem respiratory chemosensory nuclei (Kinney *et al.* 2001; Nattie & Kinney 2002).

Neonatal rats and mice that were exposed to nicotine during pregnancy showed hypoventilation and increased frequency of apnoea (St John & Leiter 1999; Robinson *et al.* 2002; Huang *et al.* 2004; Eugenin *et al.* 2008). Pre-natal nicotine also impairs hypoxia-induced autoresuscitation from primary apnoea in neonatal rats (Fewell *et al.* 2001) and hypoxia- or hypercarbia-induced ventilatory reflexes in neonatal mice, awakening rats and sleeping lambs (St John & Leiter 1999; Hafstrom *et al.* 2002; Huang *et al.* 2004; Simakajornboon *et al.* 2004; Eugenin *et al.* 2008).

Recently, using brainstem–spinal cord preparations, we have shown that pre-natal nicotine reduces central respiratory chemosensitivity in neonatal mice (Eugenin *et al.* 2008). Since pre-natal nicotine can affect the expression of acetylcholine receptors (Slotkin 1998), a reduction in central chemoreception might be due to changes in cholinergic chemosensory drive. Previous results using the 'en bloc' brainstem–spinal cord preparation indicate a change in cholinergic contribution to central chemoreception (Eugenin *et al.* 2008). To explore this possibility in more detail, we studied the effects of pre-natal nicotine exposure upon the cholinergic tonic drive of fictive respiration and upon the muscarinic and nicotinic receptor contributions to the respiratory central chemoreception. We further compared the effects of nicotine on en bloc brainstem–spinal cord and slice preparations.

2. METHODS

(a) *Preparations*

Fifteen adult CF1 mice, 5–7 days pregnant, were anaesthetized with ketamine/xylazine (80/20 mg kg^{-1} i.p.; Troy Laboratories, Smithfield, Australia and Alfasan International, Woerden, The Netherlands). Under strict aseptic conditions, subcutaneous implanting of 28-day osmotic minipumps (2004, Alzet, Cupertino, CA, USA) delivering saline (controls, $n = 5$) or nicotine bitartrate (60 mg kg^{-1} d^{-1}, $n = 10$) at a rate of 0.25 µl h^{-1} was performed through an incision made between the scapulae. As described previously (Eugenin *et al.* 2008), osmotic minipumps allow one to distinguish between the effects of nicotine itself in the foetus from those caused either by the stress of daily injections (Suemaru *et al.* 1992; Houdi *et al.* 1995) or by possible foetal hypoxia–ischaemia owing to uterine vessel vasoconstriction caused by the peak of plasmatic nicotine achieved during injections. Mice were maintained in separate cages with water and food ad libitum at 22°C under a 12 L : 12 D cycle. At the end of the experiments, animals were sacrificed with an anaesthetic overdose.

(b) *Recording fictive respiration in en bloc brainstem–spinal cord preparations and brainstem slices*

In order to evaluate whether pre-natal nicotine exposure affects chemosensory responses in a reduced preparation containing the preBötzinger complex (preBötC) (Smith *et al.* 1991), experiments were performed in slices and compared with en bloc brainstem–spinal cord preparations that also contained the pre-inspiratory parafacial respiratory group (Onimaru *et al.* 2006).

Experiments were carried out in 96 newborn animals (P0–P6), anaesthetized with methophane inhalation and cooled on ice. The central nervous system was removed, decerebrated through a ponto-bulbar transection and immersed in artificial cerebrospinal fluid (aCSF) containing (in mM): 125.0 NaCl, 5.0 KCl, 24.0 $NaHCO_3$, 1.25 $KH_2PO_4 \times H_2O$, 0.8 $CaCl_2$, 1.25 $MgSO_4 \times 7H_2O$ (Sigma, St Louis, MO, USA), 30.0 D-glucose (Merck, Darmstadt, Germany) and equilibrated with $O_2 : CO_2 = 95$ per cent : 5 per cent (pH 7.40) at 4°C. For en bloc preparations, the isolated tissue constituted by the brainstem and the spinal cord was transferred to a recording chamber 2 ml in volume and superfused with aCSF at 25°C. 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}). For the slice preparation, the brainstem was mounted on agar, and a 700 µm slice containing the preBötC was obtained using a vibratome as described previously (Pena & Ramirez 2004). Slices were transferred to a 0.5 ml recording chamber with a continuous flow of 1.0–2.0 ml min^{-1}.

(c) *Electrical recording*

Spontaneous activities from C3–C5 ventral roots in en bloc preparations or from the ventral respiratory group (VRG) in slices were recorded using glass suction electrodes at 24–25°C. Under stereomicroscopic vision, the tip of the glass electrode driven by a three-axis micro-manipulator was placed in close contact with the caudal surface of the slice in the region of the VRG. Electrical signals were amplified by a low-noise differential amplifier (Grass, Model P55), integrated with a full-wave rectifier (time constant = 100 ms), displayed on an oscilloscope (VC 6041, Hitachi, Japan) and recorded and analysed with an Axoscope-Digipack 1320A AD acquisition system (Axon Instruments, Union City, CA, USA).

(d) *Acidic stimulation*

The pH of the brainstem superfusion medium (7.3 and 7.4) was obtained by equilibrating aCSF prepared with different final concentrations of sodium bicarbonate (19 and 24 mM, respectively) with 95 per cent O_2/5 per cent CO_2. Reduction in sodium bicarbonate from 24 to 19 mM was compensated by increasing the final concentration of NaCl to 130 mM 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 (Eugenin *et al.* 2006). In addition, changes in pH were obtained by switching the equilibrating gas mixture from 95 per cent O_2 and 5 per cent CO_2 (pH 7.4) to 90 per cent O_2 and 10 per cent CO_2 (pH 7.2). The tip of a micro-combination pH electrode (Model 9811, Orion, Beverly, MA, USA) was placed in the recording

chamber and connected to a pH/ion amplifier (Model 2000, A-M Systems, Everett, WA, USA) to record the pH of the superfusion medium. Before the evaluation of the effects of acidification, ventral root activity had to be regular and stable for at least 3 min.

(e) *Evaluation of cholinergic contribution to chemosensitivity and of cholinergic drive upon fictive respiration*

Cholinergic contribution to central chemosensitivity was evaluated in en bloc and slice preparations. Fictive respiration was recorded while superfusion medium was switched from pH 7.4 to 7.3 in the presence or absence of aCSF containing muscarinic acetylcholine receptor blocker, atropine 100 μM (Sigma) or nicotinic acetylcholine receptor (nAChR) blocker, hexamethonium chloride 100 μM (Sigma). After pH 7.3 stimulation, the superfusion was returned to pH 7.4 and a recovery recording was performed.

Cholinergic drive of fictive respiration was evaluated in en bloc and slice preparations, by measuring the effects of muscarinic and nAChR blockers on the basal fictive respiration. Tonic actions of endogenous acetylcholine release were evaluated through the effects of an acetylcholinesterase blocker, neostigmine 100 μM, upon fictive respiration.

(f) *Data analysis*

Neonates in each experimental group were obtained from at least three to four litters. 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 the respiratory-like rhythm, was measured from the onset to the offset of the burst. Instantaneous rhythm frequency was calculated from the reciprocal value of the cycle duration and expressed as bursts per minute. Values were expressed as mean ± s.e.m.

The statistical significance of differences induced by treatments (acidification and cholinergic drugs) was ascertained using a two-tailed p-level estimated through a Wilcoxon signed-rank test. Differences in the magnitude of responses between control and nicotine-exposed preparations were assessed with Student's t-test for independent samples. Comparison of multiple independent groups was performed using a two-tailed p-level estimated through ANOVA followed by Bonferroni *post hoc* test. Rejection of the null hypothesis was done if $p < 0.05$.

3. RESULTS

(a) *Basal activity and responses to acidification*

In control neonatal mice, spontaneous rhythmic activity recorded from en bloc preparations consisted of bursts of action potentials appearing rhythmically at a frequency of 9.7 ± 0.7 bursts min^{-1} (ranging from 3 to 18 bursts min^{-1}), with a duration of 0.91 ± 0.03 s ($n = 15$, figure 1*a*). In control slices, bursts of action potentials recorded from the surface of the VRG appeared rhythmically at a frequency of 11.3 ± 1.4 bursts min^{-1} and lasting 0.87 ± 0.03 s ($n = 8$, figure 1*b*). No significant differences in these parameters were found between en bloc and slice preparations.

Acidification of the superfusion medium induced changes in fictive respiration in control en bloc (P0–P3) and slice (P1–P6) preparations. As previously described (Infante *et al.* 2003; Eugenin *et al.* 2006), acidification of the brainstem superfusion medium from 7.4 to 7.3 reduced the amplitude of the integrated inspiratory burst and increased the frequency of the fictive respiration in en bloc preparations (figure 1*a*,*c*,*d*). In slices, a similar pattern of response was observed: an increase in respiratory frequency (figure 1*b*,*d*) and a decrease in the amplitude of the integrated burst ($p < 0.01$, Wilcoxon test). However, the reduction in amplitude was significantly lower than that observed in en bloc preparations (figure 1*c*, $p < 0.01$, unpaired t-test).

(b) *Effects of pre-natal nicotine upon basal activity and acidification responses*

Basal frequency of fictive respiration in en bloc preparations from nicotine-exposed mice was 7.0 ± 1.1 bursts min^{-1} ($n = 10$), which was lower than that found in controls ($p < 0.01$, unpaired t-test). The duration of the inspiratory burst was 0.97 ± 0.09 s (figure 2*a*) and not different from control. Contrary to en bloc preparations, slices from nicotine-exposed mice showed a higher basal frequency than that observed in control slices (14.6 ± 1.5 bursts min^{-1}, $n = 9$, $p < 0.01$, unpaired t-test, figure 2*b*). The cycle duration in slices from nicotine-exposed mice was 0.85 ± 0.04 s.

As illustrated in figure 2*c*,*d*, in en bloc preparations from nicotine-exposed mice, acidification induced a decrease in the amplitude and an increase in the frequency ($p < 0.01$, Wilcoxon test). In slice preparations, acidification induced only a frequency increase but not an amplitude decrease (figure 2*c*,*d*). The increases in the basal frequency of fictive respiration induced by acidosis (figure 2*a*,*b*,*d*) in en bloc and slice preparations from nicotine-exposed mice were similar to those observed in controls. However, the reductions in amplitude were smaller in en bloc preparations obtained from nicotine-exposed mice than those from controls ($p < 0.01$, unpaired t-test).

(c) *Cholinergic drive of fictive respiration* in vitro

To evaluate the basal tonic cholinergic drive of fictive respiration, we studied the changes in amplitude and frequency induced by muscarinic (atropine) and nicotinic (hexamethonium) acetylcholine receptor blockers. In addition, we evaluated endogenous release of acetylcholine through the effects of neostigmine, an acetylcholinoesterase blocker.

(i) *Muscarinic and nicotinic receptor blockade*

Atropine, but not hexamethonium, reduced the amplitude and frequency of basal (pH 7.4) fictive respiration

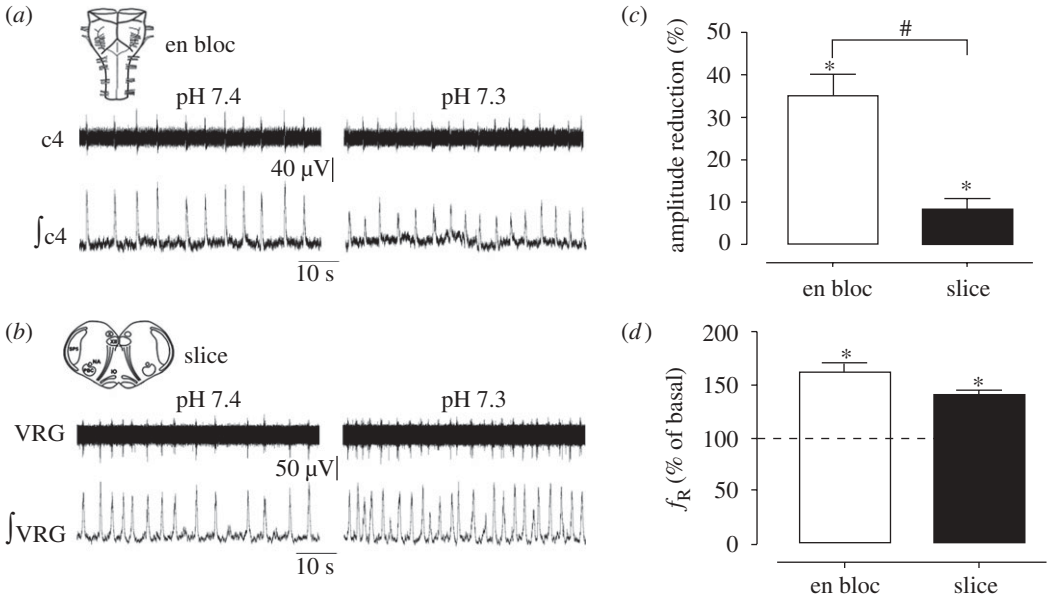

Figure 1. Effects of acidification on en bloc and slice preparations from control mice. (*a*) En bloc preparation, raw and integrated signals recorded from C4 ventral root at pH 7.4 and 7.3. (*b*) Slice preparation, raw and integrated signals recorded from the VRG at pH 7.4 and 7.3. Histograms correspond to the average of the amplitude reduction, expressed (*c*) in percentage, and (*d*) the frequency increase, expressed in percentage of basal values induced by acidification in 15 en bloc (open bars) and 8 slice (filled bars). #$p < 0.01$, unpaired *t*-test; *$p < 0.05$ respect to basal value (pH 7.4, Wilcoxon test).

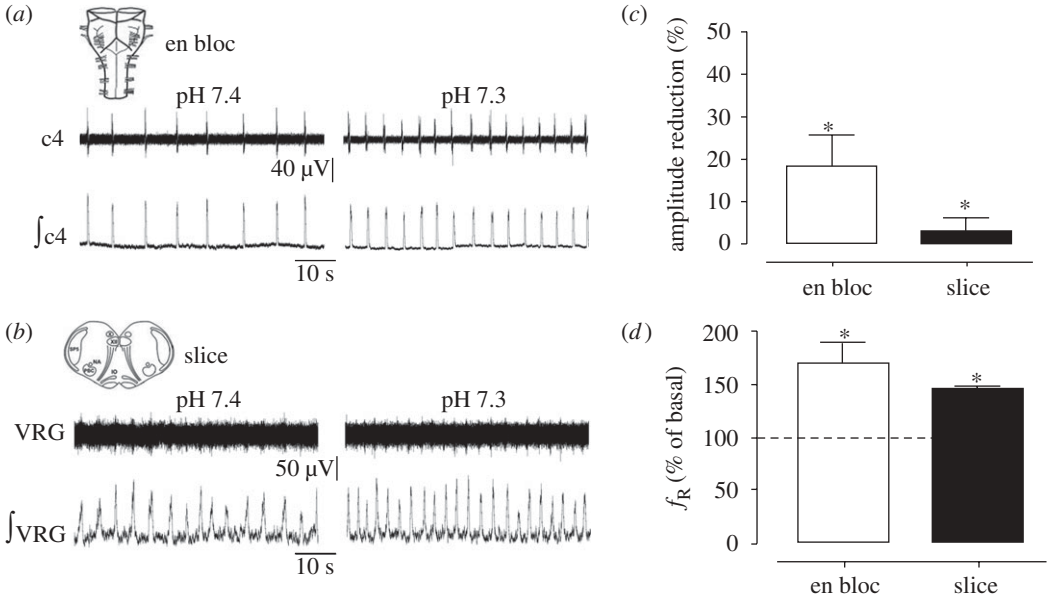

Figure 2. Effects of acidification on en bloc and slice preparations from nicotine-exposed mice. (*a*) En bloc preparation, raw and integrated signals recorded from C4 ventral root at pH 7.4 and 7.3. (*b*) Slice preparation, raw and integrated signals recorded from the VRG at pH 7.4 and 7.3. Histograms average the amplitude reduction, expressed (*c*) in percentage, and the (*d*) frequency increase, expressed in percentage of basal values induced by acidification from 10 en bloc (open bars) and 8 slice (filled bars) preparations. *$p < 0.05$ respect to basal value (pH 7.4, Wilcoxon test).

by approximately 30 per cent in en bloc preparations from control and nicotine-exposed mice ($p < 0.05$, Wilcoxon test). In contrast, neither atropine nor hexamethonium had a significant effect on fictive respiration recorded in slices from control or nicotine exposed mice (figure 3*a*,*b*).

(ii) *Acetylcholinesterase blockade*

Superfusion with neostigmine reduced the amplitude and increased the frequency of basal (pH 7.4) fictive respiration ($p < 0.05$, Wilcoxon test, figure 3*a*,*b*) in en bloc preparations from control and nicotine-exposed mice. This dual effect of neostigmine is similar to that observed with administration of carbachol, a synthetic acetylcholine agonist (data not shown). In control slices, neostigmine also increased the frequency and decreased the amplitude. But, in slices from nicotine-exposed mice, neostigmine increased only the frequency of fictive respiration (figure 3*b*).

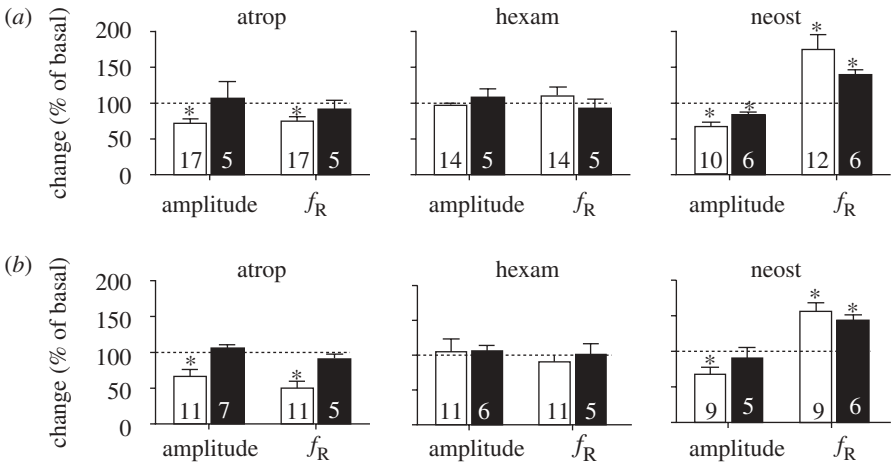

Figure 3. Cholinergic drive of fictive respiration in preparations from (*a*) control and (*b*) nicotine-exposed mice. Changes in amplitude and frequency of fictive respiration (f_R) after the application of 100 μM atropine (atrop), or 100 μM hexamethonium (hexam) or 100 μM neostigmine (neost) in en bloc (open bars) and slice (filled bars) preparations. $*p < 0.05$, when compared with the basal response (Wilcoxon test). Dotted lines indicate the basal amplitude and frequency (100%). The number of neonates is indicated inside bars.

(**d**) *Cholinergic contribution to central respiratory chemosensitivity*

In order to evaluate the cholinergic contribution to central respiratory chemoreceptory mechanisms, changes in fictive respiration induced by acidification of the superfusion medium (from pH 7.4 to 7.1) were recorded in en bloc and brainstem slice preparations from controls and nicotine-exposed mice in the absence and presence of muscarinic (atropine 100 μM) and nicotinic (hexamethonium 100 μM) acetylcholine receptor blockers.

Essentially, the contribution of muscarinic acetylcholine receptors was diminished, while that of nAChRs was increased. In control en bloc preparations, atropine, but not hexamethonium, blocked the acidification-induced reductions in amplitude and increases in frequency of fictive respiration (figures 4*a* and 5*a*, $p < 0.05$, Wilcoxon test). In contrast, in en bloc preparations from nicotine-exposed mice, hexamethonium but not atropine blocked both the acidification-induced reductions in amplitude and the increases in frequency of fictive respiration (figures 4*b* and 5*b*).

In control slices, atropine or hexamethonium blocked the acidification-induced reduction in amplitude and the increase in frequency of fictive respiration (figures 4*a* and 5*a*, respectively, $p < 0.05$, Wilcoxon test). On the other hand, in slices from nicotine-exposed mice, no significant change in amplitude was observed in the absence or presence of atropine or hexamethonium. However, hexamethonium, but not atropine, blocked the acidification-induced increase in frequency observed in slice preparations from nicotine-exposed mice.

In summary, in general terms, in control preparations, atropine, but not hexamethonium, suppressed the respiratory responses to acidification of the superfusion medium from pH 7.4 to 7.1 (figures 4*a* and 5*a*). However, in preparations from nicotine-exposed mice, atropine did 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 (figures 4*a* and 5*a*).

4. DISCUSSION

Our findings show that pre-natal nicotine exposure modifies the cholinergic contribution to central respiratory chemosensitivity. This switch from muscarinic to nicotinic receptor-based mechanisms is compatible with the known effect of pre-natal nicotine exposure of inducing downregulation of muscarinic acetylcholine receptors and upregulation of nAChRs in rat and mouse brains (Slotkin 1998). Pre-natal nicotine reduces the binding of M2 muscarinic receptors in the rat brainstem at early post-natal periods (Slotkin *et al.* 1999). In other systems, pre-natal nicotine can alter muscarinic receptor actions, either by uncoupling G-protein-dependent mechanisms in rat striatum and hippocampus (Zahalka *et al.* 1993) or by reducing mRNA of the muscarinic receptor in basal ganglia (Frank *et al.* 2001). Chronic nicotine exposure may lead to desensitization of nAChRs (Wang & Sun 2005) or their upregulation (Gaimarri *et al.* 2007). Chronic nicotine can affect selectively the number, stoichiometry, subunit composition and functionality of specific nAchRs (Van De Kamp & Collins 1994; Nguyen *et al.* 2003; Gaimarri *et al.* 2007; Walsh *et al.* 2008), especially the $\alpha4\beta2$ subtype (Perry *et al.* 1999; Gentry & Lukas 2002).

Although en bloc and slice preparations differ in their responses in controls, both kinds of preparations showed modification of the cholinergic contribution to chemosensitivity after pre-natal nicotine exposure. The most obvious difference in the pattern of responses in controls is the magnitude of the reduction in amplitude induced by acidification. This is minimal in slices, despite the similar increases in frequency observed in both preparations. Differential distribution of cholinergic receptors along respiratory

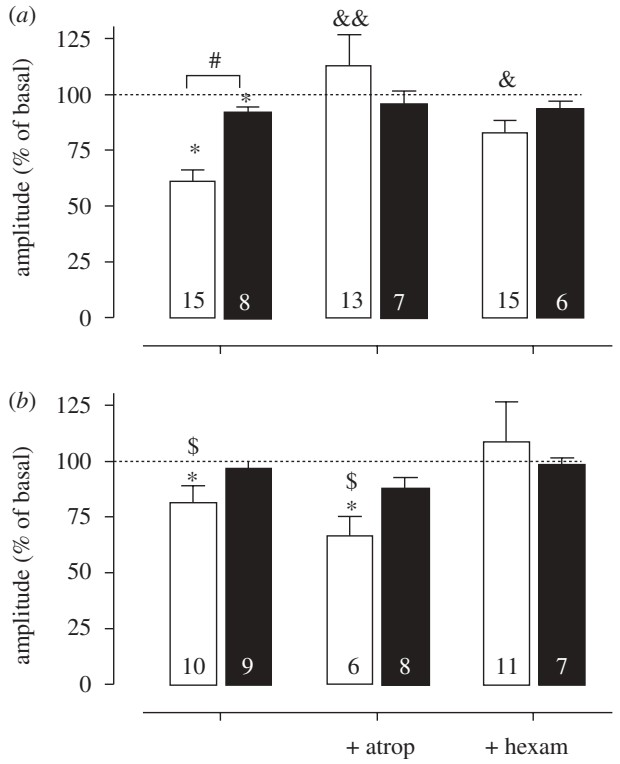

Figure 4. Changes in amplitude induced by acidification in en bloc (open bars) and slice (filled bars) preparations from (*a*) control and (*b*) nicotine-exposed mice. Preparations from P0 to P6 neonates were acidified in the absence or presence of atropine (100 μM) or hexamethonium (100 μM). $*p < 0.05$ respect to basal value (pH 7.4, Wilcoxon test); $\#p < 0.01$ between en bloc and slice preparations, ANOVA and Bonferroni post-test; $\&p < 0.05$ and $\&\&p < 0.01$ when compared with the basal condition (without antagonists, ANOVA); $\$p < 0.05$ respect to control animals, unpaired *t*-test. Changes are expressed as percentage of basal values. Bars and vertical lines represent mean and s.e.m., respectively. Dotted lines indicate the basal amplitude without acidification (100%). Number of neonates is indicated inside bars.

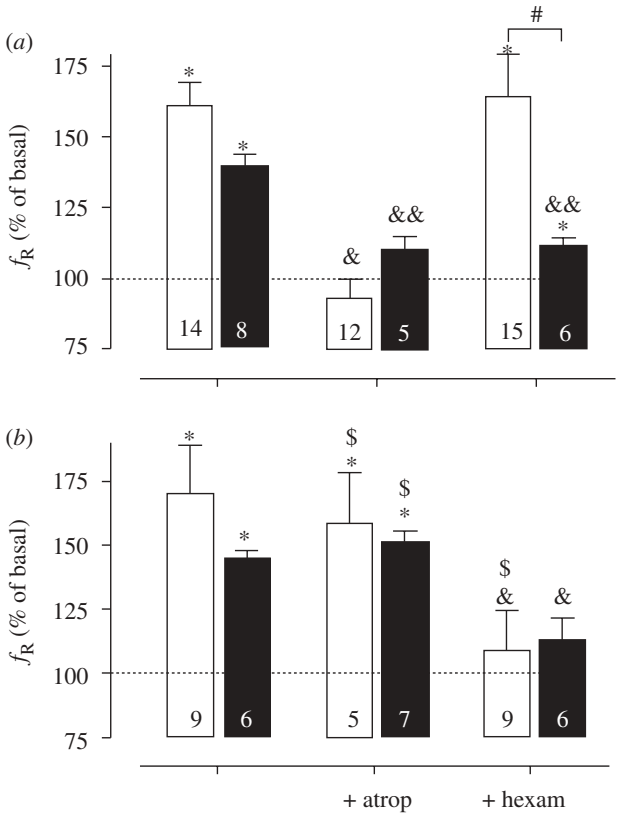

Figure 5. Changes in frequency induced by acidification in en bloc (open bars) and slice (filled bars) preparations from (*a*) control and (*b*) nicotine-exposed mice. Preparations from P0 to P6 neonates were acidified in the absence or presence of atropine (100 μM) or hexamethonium (100 μM). $*p < 0.05$ respect to basal value (pH 7.4, Wilcoxon test); $\#p < 0.05$ between en bloc and slice preparations (ANOVA and Bonferroni post-test); $\&p < 0.05$ and $\&\&p < 0.01$ respect to the basal condition (without antagonists, ANOVA); $\$p < 0.05$ respect to control animals, unpaired *t*-test. Changes are expressed as percentage of basal values. Bars and vertical lines represent mean and s.e.m., respectively. Dotted lines indicate the basal frequency without acidification (100%). Number of neonates is indicated inside bars.

brainstem nuclei may account for differential effects upon amplitude and frequency. Since the changes in frequency must rely on respiratory rhythm generators such as the preBötC, the changes in amplitude must be mostly related to other brainstem regions that are present in the en bloc preparation, but absent in slices. In agreement with this, neostigmine decreased the amplitude in en bloc but not in slice preparations.

Among the chemosensitive nuclei included in the slices, a possible target for nicotine actions is the preBötC. This chemosensitive nucleus (Solomon 2003) is crucial for generating the rhythm (Smith *et al.* 1991), and their neurons express M3 mAchR and $\alpha4\beta2$ nAchR, receptors whose activation increases the respiratory frequency (Shao & Feldman 2002; Shao *et al.* 2008). Whether these receptor subtypes are involved in central chemosensitivity has not been defined. The greatest amount of mAchR binding in the respiratory network is found in the lateral and medial parabrachial nuclei and the lateral nucleus of the solitary tract (Mallios *et al.* 1995). Interestingly, mAChRs were also found in the nuclei of the VRG (nucleus ambiguus and retrofacial nucleus) and ventral medulla (retrotrapezoid nucleus and ventrolateral medulla), which could participate in the cholinergic drive of central chemosensitivity (Nattie *et al.* 1989; Guyenet *et al.* 2008). In addition, pre-natal nicotine exposure reduces $\alpha7$ nAChRs in the forebrain and upregulates these in the brainstem and cerebellum of rats (Slotkin *et al.* 2004). It has been proposed that dysfunction of the alpha7 nAChRs can lead to impairment in the modulation of the pre-synaptic release of GABA and glutamate, which, in turn, could lead to changes in the density and/or function of post-synaptic receptors (Fregosi & Pilarski 2008). In fact, pre-natal nicotine exposure increases the inhibitory effects of GABA and glycine on preBötzinger neurons (Luo *et al.* 2004, 2007).

Our results indicate that the tonic basal cholinergic drive of fictive respiration was not affected by pre-natal nicotine. Thus, the magnitude and pattern of changes in fictive respiration induced by neostigmine and acetylcholine receptor blockers were similar in control

and nicotine-exposed preparations. At a first glance, this result appears puzzling since the central chemosensory response, which in part is mediated by cholinergic mechanisms, is decreased by pre-natal nicotine. Such disparity may reflect that cholinergic contribution to the central chemosensitivity is a low fraction of cholinergic mechanisms involved in respiratory neural control. In addition, our results show complex interactions between different cholinergic mechanisms. The prediction from neostigmine effects is that the cholinergic blockade during basal conditions should increase the amplitude and decrease the frequency of fictive respiration. Indeed, cholinergic blockade decreased both the amplitude and the frequency of fictive respiration. Access of acetylcholine to extra-synaptic receptors might account for this discrepancy. Whether adaptive mechanisms are triggered at the brainstem to counterbalance an increased nicotinic contribution with a decay in the cholinergic drive supported by muscarinic receptors is an open question.

Finally, it should be remarked that nicotine infusion alone is not a model for tobacco smoking, because nicotine is one of several chemicals in cigarette smoke that have addictive and potentially teratogenic effects (Rose 2006). In addition, minipumps do not produce intermittent infusion of nicotine as occurs in smokers. However, they allow us to study the nicotine effects on brain development, mimicking the steady-state plasma levels of nicotine ($15-45\ \mathrm{ng\ ml^{-1}}$) observed in pregnant women considered moderate smokers (Benowitz & Jacob 1984) but without confounding hypoxia and stress-derived factors associated with multiple subcutaneous injections. As well as exposure to tobacco smoke, nicotine infusion results in a similar upregulation of nAChRs in the cortex and brainstem (Slotkin *et al.* 2002). The nicotine infusion used in mice is 10 times greater than that used in rats and produces reliable levels of plasmatic nicotine around $250\ \mathrm{ng\ ml^{-1}}$ (Robinson *et al.* 2002; Eugenin *et al.* 2008). Such doses induce similar levels of nicotinic receptor upregulation in hypothalamus, hippocampus and cortex (Van De Kamp & Collins 1994) and in respiratory-related regions of the brainstem in mice (Pauly *et al.* 1991; Robinson *et al.* 2002). In addition, these doses do not affect litter size, birth weight or post-natal growth curve in mice (Robinson *et al.* 2002; Eugenin *et al.* 2008).

In conclusion, we show that pre-natal nicotine exposure decreases the central chemosensory responses and modifies the cholinergic contribution to central chemosensitivity, reducing muscarinic and increasing nicotinic commands. Dysfunction in central chemoreception and its cholinergic drive may play a role in disorders of respiratory control such as SIDS. In fact, infant victims of SIDS show a decrease in the muscarinic binding in the arcuate nucleus, which would contribute to the chemosensory drive of respiration in humans (Kinney *et al.* 1995*a*,*b*).

Experiments were performed according to the Institute for Laboratory Animal Research (ILAR) Guide for the Care and Use of Laboratory Animals and approved by the Bioethics Committee of the Universidad de Santiago de Chile.

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