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# Prenatal nicotine exposure increases hyperventilation in α4-knock-out mice during mild asphyxia

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ABSTRACT

Prenatal nicotine exposure alters breathing and ventilatory responses to stress through stimulation of nicotine acetylcholine receptors (nAChRs). We tested the hypothesis that α4–containing nAChRs are involved in mediating the effects of prenatal nicotine exposure on ventilatory and metabolic responses to intermittent mild asphyxia (MA). Using open-flow plethysmography, we measured ventilation ($\dot{V}_E$) and rate of $O_2$ consumption ($\dot{V}_{O_2}$) of wild-type (WT) and α4-knock-out (KO) mice, at postnatal (P) days 1–2 and 7–8, with and without prenatal nicotine exposure (6 mg kg$^{-1}$ day$^{-1}$ beginning on embryonic day 14). Mice were exposed to seven 2 min cycles of mild asphyxia (10% $O_2$ and 5% $CO_2$), each interspersed with 2 min of air. Compared to WT, α4 KO mice had increased air $\dot{V}_E$ and $\dot{V}_{O_2}$ at P7-8, but not P1-2. Irrespective of age, genotype had no effect on the hyperventilatory response (increase in $\dot{V}_E/\dot{V}_{O_2}$) to MA. At P1-2, nicotine suppressed air $\dot{V}_E$ and $\dot{V}_{O_2}$ in both genotypes but did not affect the hyperventilatory response to MA. At P7-8 nicotine suppressed air $\dot{V}_E$ and $\dot{V}_{O_2}$ of only α4 KO's but also significantly enhanced $\dot{V}_E$ during MA (nearly double that of WT; $p < 0.001$). This study has revealed complex effects of α4 nAChR deficiency and prenatal nicotine exposure on ventilatory and metabolic interactions and responses to stress.


## 1. Introduction

Nicotine exposure during foetal development impairs ventilation and associated responses of mammalian neonates. Prenatal nicotine exposure depresses ventilatory drive in human infants and rat pups (St.-John and Leiter, 1999; Ueda et al., 1999), delays the arousal response to hypoxia in lambs and human infants (Hafstrom et al., 2000; Parsiow et al., 2004), increases apneas in rats (Fewell and Smith, 1998) and in rats and mice, blunts the hyperventilatory responses to hypoxia and hypercapnia (Eugenin et al., 2008; Huang et al., 2010). As the main targets of nicotine, nicotine acetylcholine receptors (nAChRs) are most likely involved. Recently the use of knock-out (KO) mice, which lack a specific nAChR subunit have provided a novel approach for the study of respiratory control, providing significant insight into possible mechanisms for respiratory instability and how these can propagate and affect short and long term survival (Cohen and Lagercrantz, 2009). This approach has also yielded valuable information about the involvement of nAChRs in mediating the effects of nicotine on breathing and responses to stress and also their own role in respiratory control (Cohen et al., 2002, 2005; Dauger et al., 2004).

In mammals, the most expressed nAChR subtype in central respiratory related areas is α4β2 (Wonnacott et al., 1996). More importantly, this subtype is found in both the chemoreceptive brainstem nuclei (Duncan et al., 2008; Shao et al., 2008; Shao and Feldman, 2002) and the carotid bodies (Bairam et al., 2007; Cohen et al., 2002; Meza et al., 2012; Shirahata et al., 1998) and has the highest sensitivity to nicotine (Flores et al., 1992; Sharples and Wonnacott, 2001). Studies performed on neonatal α4-knock-in mice, expressing a hypersensitive version of the α4 subunit, have reported an excitatory role for α4-containing nAChRs on the activity of respiratory neurons *in vitro* (Shao et al., 2008; Shao and Feldman, 2002). Other work on rats and mice support a primarily inhibitory role for these receptors through their modulation of GABA (Alkondon et al., 1997; Lu et al., 1998). Additionally, genetic deletion of the β2 subunit in mice increases resting ventilation (Dauger et al., 2004) and the ventilatory response to hypoxia and also reduces the ability of exogenous nicotine to blunt the

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ventilatory and arousal responses to hypoxia (Cohen et al., 2002, 2005). These data suggest an important role for α4β2 nAChRs in respiratory control and utlimately in mediating the effects of nicotine on respiratory processes. However, at present, little is known about the role of α4-containing nAChRs in respiratory control *in vivo*.

In this study we investigate the hypothesis that α4-containing nAChRs are involved in mediating the effects of prenatal nicotine exposure on neonatal ventilatory and metabolic responses to stress. To achieve this we measured $\dot{V}_E$ of newly born (1–2 days old) and week-old (7–8 days old) nicotine treated mice in which the α4 nAChR subunit was genetically deleted or "knocked-out" (α4 KO), during intermittent exposure to mild asphyxia (MA). We chose intermittent mild asphyxia because of its clinical and developmental relevance particularly with regards to its role in sleep apnea (Cohen et al., 2012; Xie et al., 2000) and Sudden Infant Death Syndrome (SIDS) (Harper et al., 2000; Thach et al., 1991). Because of its significant contribution to respiratory responses in the neonatal rodent (Mortola, 1993; Saiki and Mortola, 1996) and the relative lack of available literature on the effect of nicotine on metabolic and ventilatory interactions we also measured $\dot{V}_{O_2}$. Examining the interactions between respiratory and cholinergic control in early life can have important clinical implications particularly for SIDS which is closely correlated with maternal smoking and abnormal respiratory function (Duncan et al., 2009).

## 2. Materials and methods

### 2.1. Experimental animals and prenatal nicotine exposure

Experiments were conducted using neonatal wildtype (WT) and knock-out (KO) mice lacking the α4 nAChR subunit (Marubio et al., 1999; Ross et al., 2000). To obtain these, overnight matings of adult WT and homozygous α4 KO mice were conducted. The presence of a vaginal sperm plug in females the next morning indicated a successful mating with a substantial increase in weight by embryonic day 10 confirming pregnancy. At embryonic day 14 a mini-osmotic pump (Alzet, model 1002 infusion rate 0.25 μl $h^{-1}$) containing water (sham) or a solution of nicotine bitartrate calculated to deliver a dose of nicotine free base equivalent to 6 mg $kg^{-1}$ $day^{-1}$ for 14 days, was subcutaneously implanted in the interscapular region of WT or α4 KO pregnant dams (Cohen et al., 2005). It should be noted that the pump marketed as a 14 day infusion device actually takes 17.5 days to be completely exhausted (information supplied by the supplier) (Slotkin et al., 2007; Xu et al., 2001), thus neonatal pups were receiving nicotine in the womb from embryonic day 14 until parturition and after that *via* maternal milk *at least* until postnatal day (P) 9 (Narayanan et al., 2002).

All experimental procedures were approved in advance by the Institution's Animal Ethics Committee. All mice were housed in the animal house and were maintained under a 12 h:12 h light–dark cycle at 22 °C unless required for an experiment. On the day of the experiment, the dam and her litter were transported to the laboratory where they were maintained under laboratory conditions (22 °C and light–dark cycle of approximately 14 h:10 h) and provided with water and standard dry food pellets *ad libitum* until experiments were completed. A total of 6 pups were studied from each group (WT/α4 KO ± nicotine) at P1-2 and P7-8. Each pup was not used more than once. Weights ranged between 1.0–2.5 g at P1-2 and 3.5–6.2 g at P7-8. These ages were selected because they encompass a period during which neonatal mice undergo rapid developmental changes in cholinergic function, chemoreception and thermoregulation. During this period the concentration of α4-containing nAChRs decreases (Bairam et al., 2007; Zhang et al., 1998, 1990), the hypoxic response of peripheral chemoreceptors is developed (Bamford et al., 1999; Carroll et al., 1993) and thermogenesis is improved (Cassin, 1963; Lagerspetz, 1962). Moreover it has been shown that at these ages neonatal mice are vulnerable to the effects of prenatal nicotine exposure and the development of these processes altered or impaired (Eugenin et al., 2008; Fewell and Smith, 1998). Thus we selected age groups which would better reveal the sequence of developmental aberrations caused by nicotine at different stages of development.

### 2.2. Experimental design and protocol

Prior to experimentation, pups were fitted with a 48G thermocouple inserted 4–5 mm into the rectum, held in place by a small drop of cynocrylate glue on the base of the tail for measurement of body temperature ($T_b$). A facemask constructed from a short piece of polyethylene tubing with a diameter roughly matching the snout of the pup and with minimal dead-space was fitted and sealed around the nostrils and mouth of the pup with the help of a small quantity of non-toxic removable Polyether material (Impregum F, ESPE); the other end of the tube protruded through a 10 ml syringe gasket. The gasket with the mask attached was placed within the barrel of the 10 ml syringe in such a way that the mask connected to a small head chamber into which the pup breathed; the remainder of the syringe was cut away to form a cradle on which the pup rested. The 10 ml syringe arrangement was subsequently sealed into a water-jacketed glass chamber (volume 72 ml) which was sealed at either end with 50 ml syringe gaskets; the head chamber protruding through one gasket and connected with a T-piece to a circuit through which air and various gas mixtures could be delivered to the head chamber. The thermocouple for measuring $T_b$ together with one to measure chamber temperature ($T_a$) passed through the other gasket (Fig. 1). A water bath connected to the water jacket chamber maintained $T_b$ at 32–33 °C (Dauger et al., 2004) which corresponded to $T_b$'s measured in the nest. Reciprocal $T_a$'s for these, which were also recorded in the nest ranged between 29 °C and 32 °C depending on age and genotype (Table 1). The gas flowing through the head chamber could be delivered by one of two flow balanced circuits; a flow of 30 ml $min^{-1}$ being maintained in each circuit by a tandem roller pump (Masterflex® easy-load, model 7518-10). One circuit delivered room air (normoxia, 21% $O_2$) while the other delivered mild asphyxia (10% $O_2$, 5% $CO_2$, balance nitrogen). In each circuit, pressure fluctuations due to the pump were removed by use of a pressure buffer compartment (volume = 2000 ml) prior to delivery to the head chamber. A timed solenoid, controlled through Powerlab (Powerlab/800, ADInstruments, Bella Vista, AU) selected the desired circuit. The time constant for 99% equilibration (within ±0.0001%) following a change of gas was 18 s, therefore the system reached equilibration for the new gas within 90 s.

After the desired gas mixture exited the compartment it passed through a small drying column (Drierite, Hammond Drierite Co.; volume = 5 ml) and then through $O_2$ and $CO_2$ gas analyzers (ML205 Gas analyser, ADInstruments). Rates of $O_2$ consumption and $CO_2$ production ($\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$, respectively) were determined from the gas flow through the head chamber and the difference in incurrent and ex-current fractional concentration of $O_2$ and $CO_2$ after taking into account respiratory quotient ($RQ \equiv \dot{V}_{CO_2}/\dot{V}_{O_2}$) related errors (Frappell et al., 1992). The head chamber could be bypassed, with the use of a solenoid, in order to check gas baseline values.

Breathing pattern was measured using a pneumotachograph connected to a bridge amplifier (MacLab, ADInstruments) which was placed in the in-current line just prior to the head chamber. The recorded signal from the pneumotachograph was electrically zeroed against gas flow such that the detection and measurement of flow oscillations due to breathing was achieved. The integral of flow provided volume which was calibrated by injecting and