

## Chronic nicotine induces growth retardation in neonatal rat pups

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Received 17 May 2005; accepted 13 July 2005

### Abstract

In the United State, 20% of pregnant women smoke. One of the most consistent adverse outcomes is reduced birth weight in the off-spring. Animal studies using chronic nicotine, the major psychoactive tobacco ingredient, have shown conflicting results, questioning the role of nicotine in growth retardation. To evaluate the direct effects of nicotine during a period equivalent to the human third trimester, we developed an oral gastric intubation model using neonatal rat pups. Nicotine (6 mg/kg/day) was dissolve in milk-formula and delivered during three feedings daily from postnatal day (P)1 to P7. Nicotine immediately and significantly [ $P < 0.05$ ] decreased weight gain per day (WGD) by 13.5% ( $\pm$  1 day after onset of treatment in both genders and throughout the treatment period. This resulted in significantly lower body weight at P4 and P5 in male and female pups, respectively. After nicotine withdrawal, WGD returned to control level within 1 day, whereas total body weight recovered by P18. There were no long-term consequences on body weight or growth pattern in either gender. The nicotinic acetylcholine receptor (nAChR) antagonist dihydro- $\beta$ -erythroidine (DH $\beta$ E) reversed nicotine's effects on WGD suggesting an involvement of heteromeric  $\alpha 4\beta 2$ , whereas methyllycaconitine (MLA) an antagonist for the homomeric  $\alpha 7$ -type receptor was ineffective.

The immediate decrease of growth in neonatal pups suggests that nicotine's effect on birth weight results from direct anorexic rather than indirect effects due to placental dysfunction or increased fetal hypoxia. The postnatal oral gastric intubation model seems to accurately reflect the direct effects of nicotine in neonates.

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**Keywords:** Pregnancy; Body weight; Growth; Nicotinic receptor; Gastric intubation model

### Introduction

In the United State, 20% of pregnant women smoke resulting in about 800,000 babies exposed to tobacco ingredients in utero being born annually in the US alone (Kirchengast and Hartmann, 2003; NIDA Info fax, #13568). Maternal smoking is associated with increased perinatal morbidity including increased risk of Sudden Infant Death Syndrome (SIDS) (Haglund and Cnattingius, 1990; Mitchell et al., 1993), neuropsychiatric disorders including attention deficit hyperactivity disorder (ADHD), conduct disorder and substance abuse, as well as a lower IQ (Ernst et al., 2001; Hellstrom-Lindahl and Nordberg, 2002; Batstra et al., 2003;

Fried et al., 2003), but the most consistent finding is reduced birth weight in full-term infants (Kirchengast and Hartmann, 2003; Nash and Persaud, 1988; Olsen, 1992; Vik et al., 1996). Full-term children born to heavy smokers weight on average 377 g less at birth (Wang et al., 2002) mainly due to the effects of tobacco exposure during the third trimester, a period particularly sensitive with regard to birth weight (Fried and O'Connell, 1987).

Several studies have revealed that direct and indirect mechanisms could contribute to the effects of maternal smoking on fetal growth retardation (Hafstrom et al., 2004; Bush et al., 2000). In heavily smoking mothers, placental villi demonstrate atrophic and hypovascular changes and reduced placental weight, which compromise placental function and contribute to fetal growth retardation (Mochizuki et al., 1984, 1985; Vogt Isaksen, 2004). Nicotine, the principal psychoactive component in tobacco activates maternal peripheral nicotinic acetylcholine receptors (nAChRs) which results in increased

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catecholamine release and subsequently leads to vasoconstriction and reduced placental blood flow, causing fetal hypoxia which could further compromise fetal growth (Ernst et al., 2001). Nicotine also penetrates through the placental barrier and concentrates in fetal tissue at an equal to or greater level than in maternal tissue where nicotine can directly interact with peripheral and central nAChR which could also affect fetal outcome (Dempsey and Benowitz, 2001; Luck et al., 1985). Animal models have been used to study short- and long-term consequences of developmental nicotine exposure in an attempt to link nicotine to the observed adverse effects found in babies whose mothers smoked during pregnancy. The results confirmed several behavioral findings such as enhanced locomotor activity, neurobehavioral deficits and impaired cognition (Sobrian et al., 2003; Tizabi et al., 2000; Ajarem and Ahmad, 1998; Abdel-Rahman et al., 2005). However, although, nicotine's anorexic properties are well documented for adults (Jo et al., 2002; Guan et al., 2004), a link between gestational nicotine and lower birth weight has not yet been established.

Chronic nicotine exposure in prenatal models has been found to (a) decrease birth weight (Cutler et al., 1996), (b) affect female off-spring only (Vaglenova et al., 2004), (c) affect male off-spring only (Peters and Ngan, 1982; Peters et al., 1979) or (d) result in no change in birth weight (Abreu-Villaca et al., 2004; Levin et al., 1993; Pauly et al., 2004; Chen and Kelly, 2005; Sheng et al., 2001). It is possible that developmental growth retardation, as seen in humans, is a consequence of a combination of direct and indirect effects of smoking or other tobacco ingredients besides nicotine. However, in prenatal models, variability of neonatal weight between litters independent of nicotine treatment makes it harder to establish a statistically significant drug effect. Furthermore, different treatment protocols vary based on routes of drug administration, nicotine concentration or treatment duration, which could differentially effect growth. In addition, prenatal exposure models apply nicotine via the dam, often using minipumps or nicotine containing pellets resulting in continuous drug release. This routine of maternal nicotine exposure leads to steady-state blood levels for nicotine, without the oscillation in blood nicotine concentrations typically seen in smokers. Continuous nicotine exposure desensitizes nAChRs, which renders them unresponsive to the effects of nicotine (Paradiso and Steinbach, 2003).

Another possible explanation for this discrepancy is that prenatal rodent exposure models do not address the effects of nicotine on growth during the human third trimester equivalent, a developmental period most strongly correlated with decreased birth weight in humans (Fried and O'Connell, 1987). The first postnatal week in rats corresponds more closely to this time window in terms of growth pattern and brain development, it is also referred to as the brain growth spurt period (Dobbing and Sands, 1979). Therefore, neonatal exposure models would better address the effects of nicotine on growth during late gestation in humans. In addition, postnatal models have several advantages over prenatal models such as, no indirect effects of nicotine due to exposure

of the dam, exact amount of nicotine can be delivered to the pups tailored to their individual weight, littermates can be used as controls, and intermittent drug administration resembling smoking behavior of pregnant women. A few studies have looked at the effects of chronic neonatal nicotine (Slawecki et al., 2000; Narayanan et al., 2002; Thomas et al., 2000; Girard et al., 2001; Eriksson et al., 2000) and reported on nicotine's effects on body weight (Narayanan et al., 2002; Slawecki et al., 2000; Thomas et al., 2000), but found no impact of chronic nicotine on growth. Two of these studies used an artificial rearing protocol and gastrostomy to deliver milk formula with or without nicotine to rat pups from postnatal days 4–9. This treatment resulted in reduced body weight gain for both control and nicotine-treated pups in comparison to non-handled suckling controls, possibly due to suboptimal nutrient supply and/or increased stress due to maternal deprivation suggesting interference with growth in both treatment groups (Slawecki et al., 2000; Thomas et al., 2000). Narayanan et al. (2002) applied nicotine via maternal milk, a protocol that prevents intra-litter controls and has less control over individual dosing. In addition, pups were cross-fostered which by itself can cause stress to the animals (Vaglenova et al., 2004).

In this study, we used a neonatal chronic nicotine exposure model similar to the one used to study the impact of binge alcohol exposure in postnatal animals (Hsiao et al., 2001) to address the question if neonatal nicotine exposure influences growth and has any long-term consequences on body weight. The model took advantage of oral intubation to deliver nicotine in controlled individualized doses to rat pups. It minimized maternal deprivation by keeping the pups with the dam except for brief periods on treatment days and allowed a period for nicotinic receptors to recover from desensitization.

## Materials and methods

### Animals

Timed-pregnant Sprague–Dawley rat dams purchased from Harlan (Houston) arrived on gestation day 14 and were housed under standard condition at the College of Medicine's Animal Care Facility according to the rules of Texas A&M University Laboratory Animal Care Committee. The day the rat pups were born was considered as postnatal day 0 (P0). On P1, litters were culled to 8 pups per biological dam. Pups without obvious birth deficit were randomly assigned to different treatment groups with four animals per treatment, thus, two different treatment groups per litter. A minimum of 4 litters (32 animals) were used per experiment. The studies on the effects of treatment and antagonists used an independent cohort of animals derived from different litters. To determine the short- and long-term effects of early postnatal nicotine, a total of 10 litters were treated, of those 6 and 4 litters were allowed to grow to P14 and P53, respectively, while continuously being weighted. Pups remained with the dam until weaning on P21.

### Drug administration

The oral gastric intubation model used in this study for nicotine administration has been described previously (Hsiao et al., 2001). The manual oral gastric tubing was made with a PE10 plastic tube tipped with a short segment of soft silicon tubing (0.64 mm o.d.). During treatment, all eight pups from one litter were kept in a stainless steel tray padded with paper towel on a heating pad to keep them at 34–37 °C. Pups were treated three times per day, 4 h apart (09:00, 13:00, 17:00 h). The nicotine treatment group received 2 mg/kg/dose nicotine (Sigma N3876), to reach the total amount of 6 mg/kg/day from P1 to P7. Pups were weighted every morning and milk formula (Enfamil® with iron) was made fresh daily. The volume [ml] of milk the pups received was calculated as 1/36 of the individual body weight [g] (body weight [g]/36=ml). Control pups were intubated without milk. After treatment, all 8 pups were returned to the dam. Nicotinic receptor antagonists dihydro- $\beta$ -erythroidine (DH $\beta$ E; Sigma D-149) 6 mg/kg/dose (18 mg/kg/day), and methyllycaconitine (MLA; Sigma M-168) 10 mg/kg/dose (30 mg/kg/day) were given in formula with or without nicotine. The concentrations for the antagonists were based on results previously published by others (Blondel et al., 2000; Turek et al., 1995).

### Statistical analysis

Data were expressed as means $\pm$ S.E.M. For weight gain per day (WGD) and body weight, a three-factor ANOVA was conducted with treatment (control, nicotine), gender (male, female), and time (postnatal day) as main effects. A univariate general linear model approach was used for analysis and hypothesis tests: (a) because the number of cases decreased over time; and (b) the variance generally increased with time as the animals gained weight. Following the standard estimation of main effects and interactions, linear contrasts on the model parameters were used to test the significance of either treatment or gender effects in three specific time-periods: postnatal days 1–9; postnatal days 10–23; and postnatal days 37–53. When supported by the ANOVA, post-hoc analysis was performed to test differences between selected pairs of means, with  $p < 0.05$  defined as significant.

To test differences between growth rates, data were fitted to the linear model:  $\log(\text{wt}) = a_0 + a_1 \cdot \text{day}$ . The slope,  $a_1$ , (i.e. growth rate) was either constrained to a common best-fit value for the groups being tested, or allowed to vary individually by group.  $F$  ratios (Motulsky and Ransnas, 1987) were constructed to test whether the null hypothesis of no difference between slopes could be rejected.

Data of unhandled, intubation and intubation with milk were conducted by repeated two-way ANOVA with postnatal day and treatment as two between-subject factors. Data of DH $\beta$ E and MLA or brain weight were computed by one-way ANOVA with nicotine treatment as a between factor followed by Fisher's Least Significance Difference (LSD) for post hoc analysis.  $P < 0.05$  was defined as significant.

## Results

### Intubation procedure does not alter growth

Nicotine was dissolved in milk formula to reduce stomach irritation and delivered directly into the stomach; whereas controls were sham intubated but did not receive extra milk formula in an effort to offset potentially large differences in weight gain between treatment groups. To determine if the treatment procedure or the extra formula affected weight gain or brain weight, non-handled controls, pups receiving an extra feeding of milk-formula via intubation and pups intubated but without the extra feedings were compared with regard to body weight, WGD and brain and cerebellar weight measured on P8, 16 h after the last treatment. No significant differences were found between either treatments with regard to total body weight, WGD, whole brain or cerebellar weight (Fig. 1A, B, C, D).

### Nicotine reduces body weight

The initial body weight at P1 did not differ among treatment groups and no differences between male or female pups were detected. (Fig. 2B, D). Nicotine treatment was initiated on P1 resulting in a significant decrease in WGD of 13.5% [ $F_{1, 78} = 10.121$ ,  $p < 0.002$ ] on P2. The greatest reduction of 22% compared to controls occurred on P5 (Fig. 2A). The reduced WGD resulted in significantly lower body weight compared to controls starting at P4 [ $F_{1, 78} = 8.218$ ,  $p < 0.005$ ] (Fig. 2B). Nicotine treatment caused similar changes in WGD and body weight for both male and female pups [at P2, males:  $F_{1, 42} = 5.962$ ,  $p < 0.02$ , females:  $F_{1, 34} = 4.064$ ,  $p < 0.05$ ] (Fig. 2C, D). Further analysis showed that nicotine reduced body weight earlier in males at P4 [ $F_{1, 42} = 6.144$ ,  $P < 0.02$ ] than in females at P5 [ $F_{1, 34} = 7.052$ ,  $p < 0.01$ ] (Fig. 2D). By the end of the treatment period at P8, control male and female pups weighed on average 18.4 $\pm$ 1.3 g and 18.2 $\pm$ 1.3 g, respectively, whereas nicotine-treated male and female pups were 16.5 $\pm$ 1.5 g and 16.3 $\pm$ 1.5 g, respectively, a 10.4% reduction in body weight (Fig. 2D).

### Rapid recovery of weight gain after nicotine withdrawal

After the end of nicotine treatment at P8, the rate of WGD rapidly recovered to the level of controls at P9, when significant differences between nicotine-treated and control groups were no longer detected (Fig. 3A) in either male or female pups (Fig. 3C). Body weight between nicotine treatment and control groups were comparable after P18 (P11 for nicotine males and P20 for nicotine females) as the size of the pups increased (Fig. 3 B, D). However, the initial absolute difference in weight was still apparent just before weaning, at P20, when nicotine-treated male and female pups were on average 2.3 and 2.1 g lighter than their control counter part.

### No long-term effect of nicotine on body weight and weight gain

After weaning at P21, but before puberty nicotine-treated pups grew at a similar rate as the controls with regard to WGD

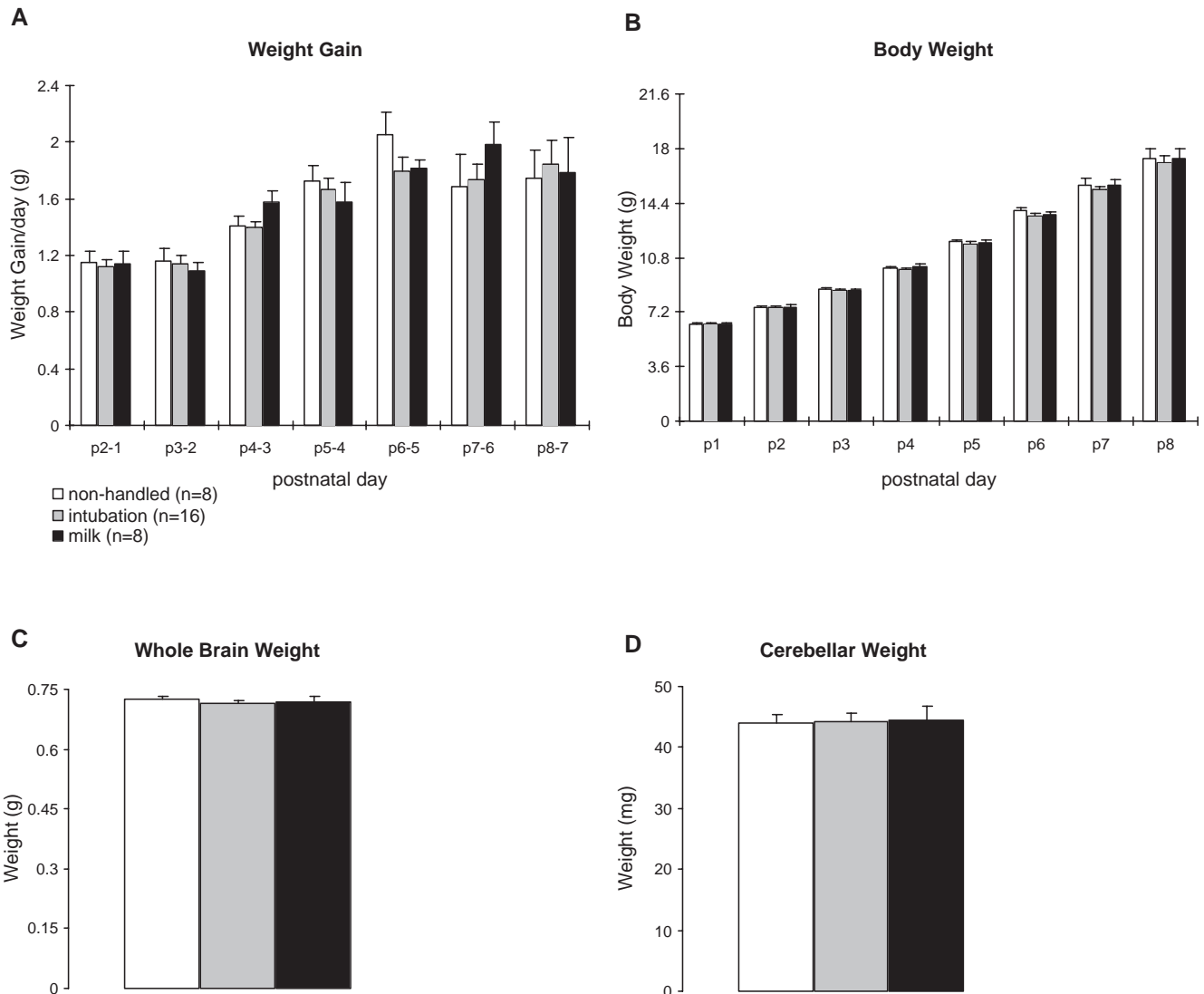


Fig. 1. Intubation procedure alone does not alter growth. Comparison of (A) weight gain per day, (B) total body weight, (C) whole brain weight, and (D) cerebellar weight at P8, in non-handled ( $n=8$ ), intubated without extra milk feeding ( $n=16$ ), and intubated plus extra milk feeding ( $n=8$ ) rat pups. Significant differences were not detected in any treatment group for any parameter using ANOVA,  $p < 0.05$ .

and body weight (Fig. 4A, B). However, during the fourth postnatal week, growth patterns started to diverge between males and females and by P30 and thereafter, body weight and WGD was significantly depended on gender in both control and nicotine-treated animals indicating the onset of puberty at the same time of development independent of treatment. On P53, body weights in young adult animals were not significantly different from their gender control group.

The overall growth pattern in control rat pups could be divided into three different growth phases (Table 1). The early growth phase ending between P9 and P10 was characterized by rapid gain in body mass independent of gender. During the second phase ending shortly after weaning, the pace of the growth had slowed and was not significantly different between genders. After a transition period, the third growth rate began after P30 and now differed significantly between genders, with males growing faster than females [control:  $F_{1,14}=19.0$ ,  $p < 0.001$ ; nicotine:  $F_{1,11}=16.5$ ,  $p < 0.002$ ]. This overall

growth pattern was not affected by nicotine treatment, although, nicotine significantly slowed growth in both genders during the first phase but had no effect on other growth periods.

#### *DH $\beta$ E reversed nicotine-induced loss of body weight*

To identify which nAChRs were involved in nicotine effects on the control of body weight, a competitive antagonist DH $\beta$ E, which inhibits heteromeric nAChRs containing  $\alpha_4$  and  $\beta_2$  subunits (Luetje et al., 1990), and MLA, which selectively blocks homomeric  $\alpha_7$  receptors (Alkondon et al., 1992) were combined with nicotine intubation. The experiment confirmed the effects of nicotine on weight gain [ $F_{1,14}=8.686$ ,  $p < 0.01$ ], DH $\beta$ E (6 mg/kg/dose) given alone did not significantly affect body weight, but DH $\beta$ E reversed nicotine-induced reduction in weight gain and was significantly different to nicotine [ $F_{1,22}=6.906$ ,  $p < 0.015$ ] (Fig. 5A). In contrast, MLA (10 mg/kg/dose)

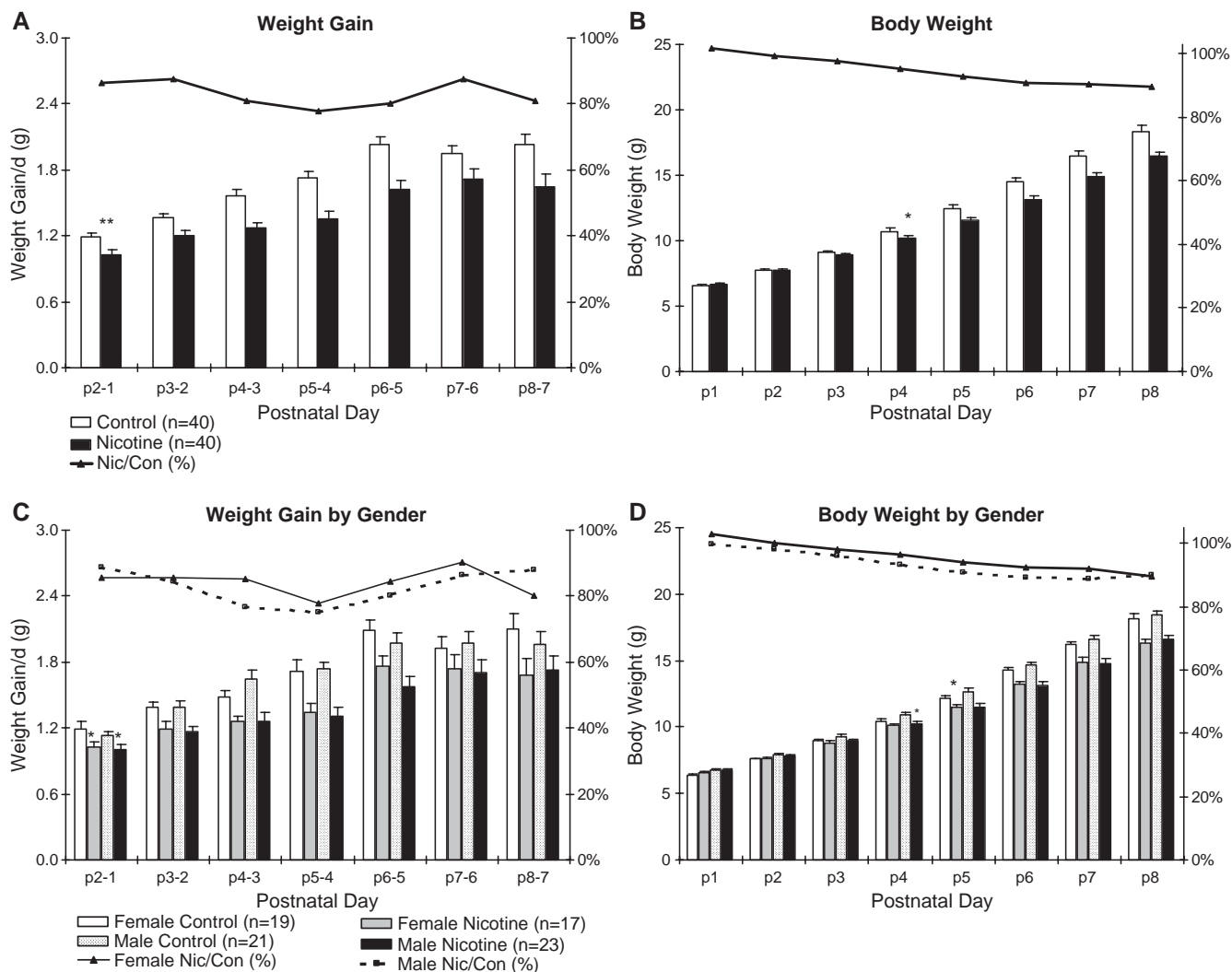


Fig. 2. Nicotine affects body mass growth. Effects of 6 mg/kg/day nicotine in rat pups treated from P1 to P7. (A) Weight gain per day, (B) total body weight, (C) weight gain per day by gender, (D) total body weight by gender. Y-axis on the left refers to bar values; Y-axis on the right indicates percent of control for nicotine-treated animals. Data were analyzed using a three-way ANOVA. \* $p < 0.05$ , \*\* $p < 0.005$  indicates first day of significant difference between treatment groups. Error bars indicate standard error.

alone did not affect body weight; nor did it inhibit nicotine-induced reduction of body weight gain (Fig. 5B).

## Discussion

### Neonatal chronic intubation model

We have developed a neonatal chronic nicotine exposure model using gastric intubation for drug delivery that allowed us to evaluate the effects of postnatal nicotine treatment on body weight and growth. The drug delivery schedule can be tailored to resemble the ups and downs in blood nicotine levels seen in smokers, as reflected in the off-spring. This approach should permit “reset periods” and result in less desensitization of nAChRs than models using continuous infusion with minipumps or implanted nicotine pellets, methods that result in continuous blood nicotine levels and resemble more “patch-like” nicotine exposure (Thomas et al., 1988). Nicotine, at the dose given, did not cause seizures and did not seem to interfere with neonatal

behavior. No obvious differences in suckling behavior were detected and pups from both groups latched on to the dam and had milk in their stomach before each treatment. In addition, there is no need for cross-fostering since the dam is not treated with the drug and maternal separation was kept to a minimum. This should greatly reduce stress in the neonates. Although, stress hormone levels were not measured, the similar growth between intubated and non-handled controls is an indication for the overall well-being of the pups. In contrast, in the artificial rearing, gastrostomy model (pup-in the cup) controls grow slower than suckle controls indicating suboptimal rearing conditions (Sla- wecki et al., 2000; Thomas et al., 2000). Thus, this oral gastric intubation model seems to be adequate to study the effects of nicotine during the third trimester equivalent in rodents.

### Short-term effects of nicotine

The major finding of this study is that nicotine results in an immediate, gender independent, significant reduction of body

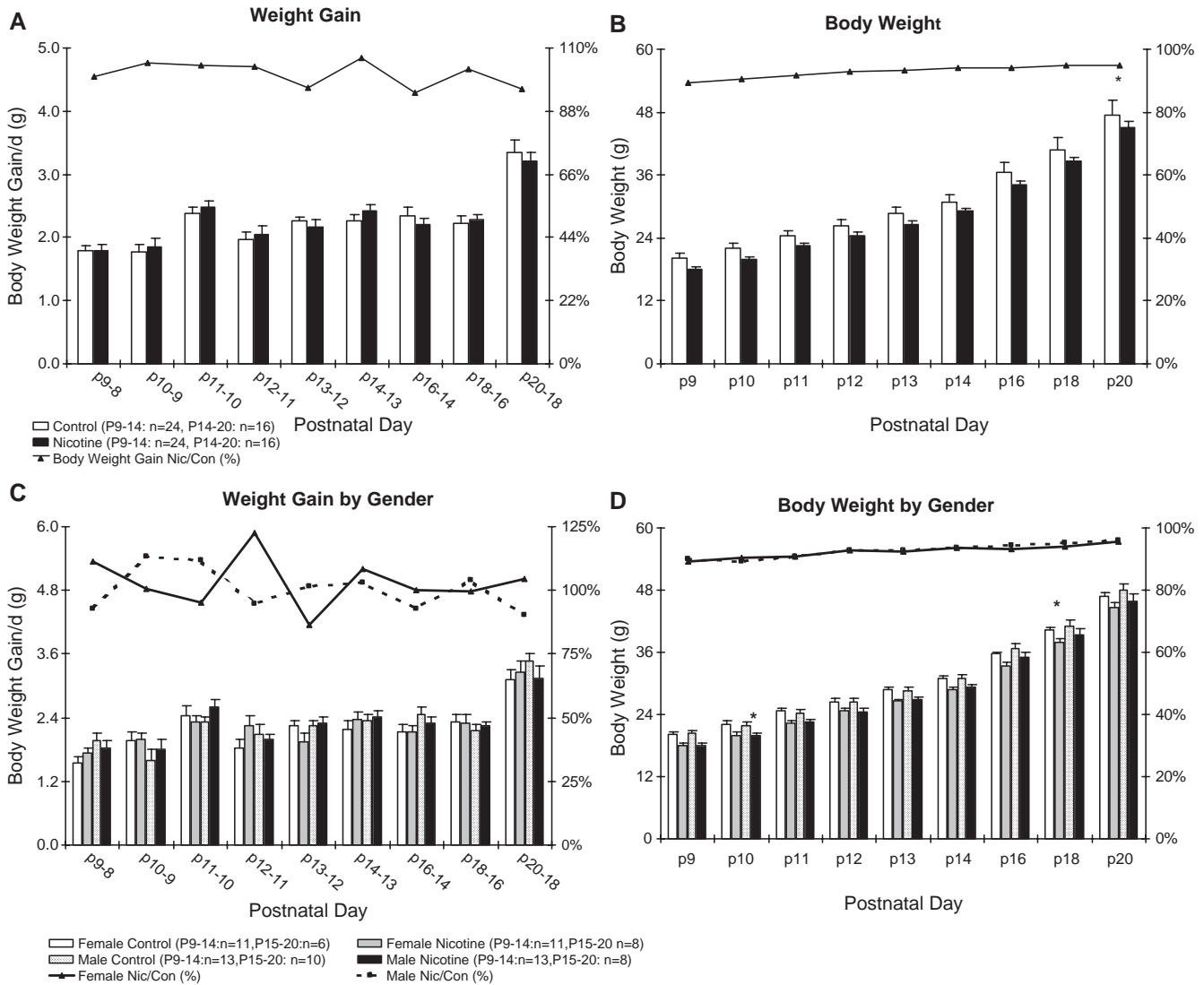


Fig. 3. Rapid recovery after nicotine withdrawal. (A) Weight gain per day, (B) total body weight, (C) weight gain per day by gender, (D) total body weight by gender, in rat pups treated with or without nicotine 6 mg/kg/day nicotine from P1 to P8. Y-axis on the left refers to bar values; Y-axis on the right indicates percent of control for nicotine-treated animals. Data were analyzed using a three-way ANOVA. \* $p < 0.05$  indicates first day significant differences between treatment groups were no longer apparent. Error bars indicate standard error.

mass growth in neonates, while nicotine is administered. Thus, nicotine may exert similar anorexic effects in developing animals, as has been well documented in adults (Schechter and Cook, 1976; Saah et al., 1994; Li et al., 2003). The results strongly suggest that nicotine is a major contributing factor to the reduction in birth weight consistently seen in babies of smoking mothers (Ernst et al., 2001). This effect could be especially important with regard to nicotine replacement therapy when considering nicotine patches or gum to reduce smoking in pregnant women (Coleman et al., 2004). In this study, a relatively high dose of nicotine (6 mg/kg/day) was used, believed to create blood nicotine levels corresponding to smokers who consume 2 packs of cigarettes per day (Murrin et al., 1987). Thus, it is possible, that lower doses would have fewer effects on growth retardation, which could explain why several studies were unable to detect reduced birth weight in prenatally treated pups. It is surprising, though, that even studies using similar doses found no or inconsistent effects

with regard to birth weight (Abreu-Villaca et al., 2004; Chen and Kelly, 2005; Vaglenova et al., 2004). However, the first 10 days of postnatal life is a period of rapid weight gain (see Table 1), equivalent to the third trimester in humans (Dobbing and Sands, 1979), when fat depots are built; and a period which is particularly sensitive to the effects of smoking (Fried and O’Connell, 1987). In adult rats, nicotine-induced weight loss is associated with changes in fat composition, whereas protein and water content is not affected (Winders and Grunberg, 1990), indicating that nicotine interferes with fat storage. Thus, nicotine in prenatal rodent exposure models might have little effect on body weight due to the lower levels of body fat in prenatal animals, whereas it can greatly interfere with weight gain during early postnatal development, when in addition to overall body growth fat depots are built. In addition, in our hands, inter-litter variability with regard to birth weight was significant (data not shown), making it difficult to establish a nicotine effect in prenatal models.

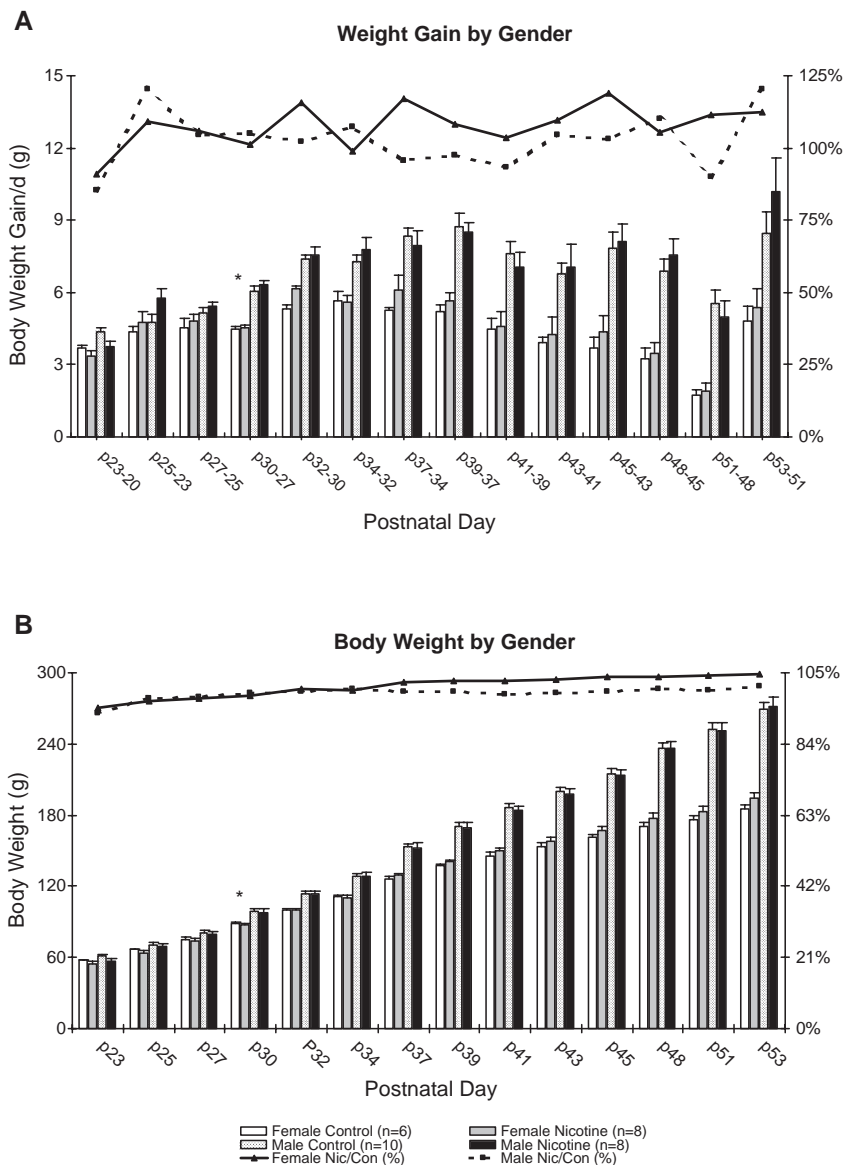


Fig. 4. No long-term effect of neonatal nicotine exposure on body weight. (A) Weight gain per day by gender, (B) total body weight by gender, in rat pups treated with or without nicotine 6 mg/kg/day nicotine from P1 to P8. Y-axis on the left refers to bar values; Y-axis on the right indicates percent of control for nicotine-treated animals. Data were analyzed using a three-way ANOVA. \* $p < 0.05$  indicates onset of significant difference between genders. Error bars indicate standard error.

*Mechanisms of nicotine-induced growth retardation*

The mechanism by which nicotine induces weight reduction in adults is not well understood. It could be a combination of

Table 1  
Growth rates of rat pups

	Control		Nicotine	
	Males	Females	Males	Females
Rate 1*	0.0606	0.0631	0.0541	0.0553
Rate 2	0.0337	0.0310	0.0347	0.0327
Rate 3**	0.0147	0.0100	0.0153	0.0108

Rate =  $k/\text{body weight}(\text{day}) = \exp(k * \text{day})$ .

Rate 1 from P1 through P9: \* nicotine male and female pups significantly different from controls,  $p < 0.005$ .

Rate 2 from P10 through P23.

Rate 3 from P37 through P53: \*\* significant gender difference,  $p < 0.005$ .

reduced food intake (Blaha et al., 1998; Guan et al., 2004), increased energy expenditure (Grunberg et al., 1988) or a combination of both (Bishop et al., 2004). Feeding behavior and energy homeostasis are regulated by complex interactions between peripheral and central mechanisms (reviewed in Jo et al., 2002; Schwartz et al., 2000). Nicotinic receptors are strategically located to interfere with both peripheral and central sites involved in the regulation. At central sites, nicotine could control food intake and energy expenditure through direct activation of nAChRs in the hypothalamus, an area critical for regulation of feeding and energy metabolism (Schwartz et al., 2000) and indirectly through activation of presynaptic receptors regulating the release of various neurotransmitters. Nicotine acting presynaptically on extrinsic projections to the hypothalamus increases levels of serotonin and dopamine (Yang et al., 1999; Miyata et al., 1999) which

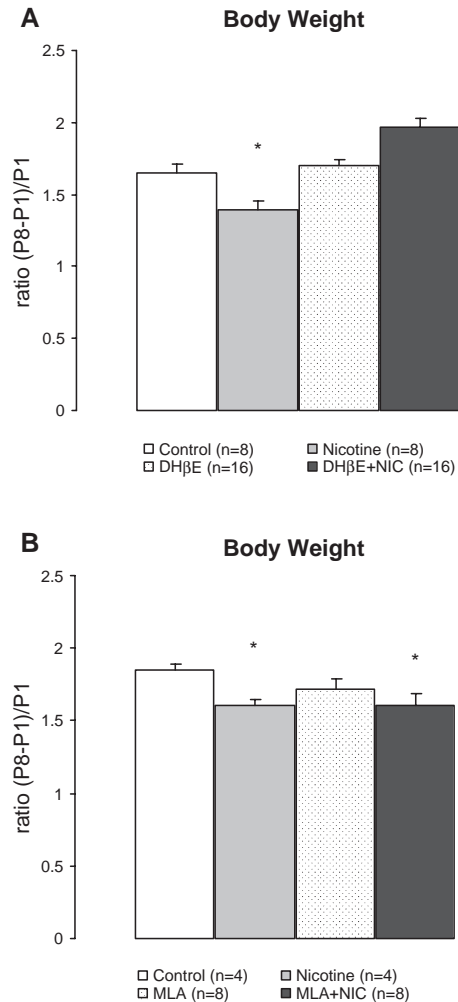


Fig. 5. Effects of nAChR antagonists on total body weight at P8. (A) Heteromeric receptor antagonist DHβE (18 mg/kg/day) and (B) homomeric receptor antagonist MLA (30 mg/kg/day) were administered from P1 to P7 alone (spotted columns), or coadministered with nicotine (6 mg/kg/day, solid black columns) and compared to control (white columns) and nicotine alone (6 mg/kg/day, solid grey columns). Total weight gain (P8–P1 body weight) was normalized to initial body weight at P1: \* $p < 0.05$ , significantly different from control; the number of animals is indicated in parentheses.

could cause reduced food intake. In adult animals, nicotine also induces modifications in a variety of feeding-related systems (Li et al., 2000) including altered expression of neuropeptide Y (Frankish et al., 1995), gastrin and CCK (Chowdhury et al., 1989) and insulin (Saah et al., 1994). These changes could be the direct result of postsynaptic activation nAChR by nicotine, and could increase energy expenditure in adults. Similar mechanism could be responsible for nicotine's effect on growth retardation in neonates because nAChRs are expressed in pre- and postnatal rat (Naeff et al., 1992; Adams et al., 2002) and human embryonic brain (Cairns and Wonnacott, 1988) and are functional during development (Leslie et al., 2002). Reduced milk consumption in response to nicotine cannot be ruled out, as it is not possible to measure the caloric intake between treatment groups, but no obvious differences in nursing behavior were detected.

To determine if nAChRs are involved in nicotine-induced weight reduction, we used the nicotinic antagonists DHβE and

MLA to block heteromeric or homomeric receptors, respectively. Nicotine-induced reduction in body weight gain could be reversed by DHβE but not MLA. This is a clear indication for the involvement of heteromeric nAChRs in nicotine's effects. Heteromeric receptor binding sites are located in the hypothalamus (Pabreza et al., 1991) where they regulate neuronal activity (Jo and Role, 2002). In addition, alpha and beta nAChR subunits are expressed in catecholaminergic neurons (Azam et al., 2002; Winzer-Serhan and Leslie, 1997; Gallardo et al., 1997) where they have been implicated in regulating transmitter release (Yang et al., 1999; Miyata et al., 1999; Leslie et al., 2002). Thus, weight regulation by an action of nicotine through pre- and postsynaptic heteromeric receptors is possible.

In contrast, MLA seems to have no or very little effect on weight gain. Thus, either  $\alpha 7$  nAChRs which are expressed in the hypothalamus (Seguela et al., 1993; Meeker et al., 1986) are not involved in weight regulation or the concentration of MLA was too low to effectively block activation of homomeric receptors by nicotine. In this study, a relatively high concentration was used to overcome the modest bioavailability of oral MLA, which should have resulted in measurable, but low brain levels (Turek et al., 1995). Since only nanomolar concentrations are needed to effectively antagonize  $\alpha 7$  receptors (Wonnacott et al., 1993) and because of the prolonged half-life after oral administration, MLA should have blocked  $\alpha 7$  nAChRs. However, it cannot be ruled out that the MLA concentration used in this study failed to antagonize nicotine's effects. On the other hand, studies have shown that  $\alpha 7$  homomeric receptors play only a minor role in nicotine-mediated presynaptic catecholamine release (Matta et al., 1995; Cao et al., 2005) and that neuronal activity in hypothalamic neurons is unaffected by an  $\alpha 7$ -selective antagonist (Jo and Role, 2002), indicating that  $\alpha 7$  would not regulate weight through either presynaptic or postsynaptic mechanisms. Thus, evidence points to heteromeric nAChRs to mediate the anorexic properties of nicotine in developing animals.

#### Long-term and gender-specific effects of nicotine

Adolescent children born to smokers tend to have increased body weight and a trend is seen in boys to have earlier onset of puberty (Fried et al., 1999, 2001). Some reports using rodent models have confirmed that prenatal chronic nicotine can have long-term consequences on weight, resulting in altered body weight in adolescent and adult rats (Chen and Kelly, 2005; Vaglenova et al., 2004), and some report of gender-specific effects on weight in response to nicotine have also been published (Peters et al., 1979; Peters and Tang, 1982). In our study, significant differences between male or female pups were not detected either during treatment, immediately thereafter or long-term. In this study, chronic neonatal nicotine exposure did not produce long-term changes of body weight, overall growth pattern or onset of puberty. This discrepancy could result from the postnatal versus prenatal time of the exposure or from indirect effects of nicotine through the dam

which could cause altered hormonal status influencing the fetus.

Short-term gender differences after prenatal exposure have been detected in male (Peters and Ngan, 1982; Peters et al., 1979) or female pups (Vaglenova et al., 2004), but others did not report any significant effects on birth weight (Slawecki et al., 2000; Fredriksson et al., 2000; Girard et al., 2001; Levin et al., 1993; Peters and Ngan, 1982; Chen and Kelly, 2005). One possible explanation is that the variability in weight between litters is high. In our hands, there were significant differences in average P1 body weight among litters (data not shown), which could be explained by difference in the number of pups per litter, male to female ratio or slight variations in gestation time.

On the other hand, several groups have reported long-term gender-specific consequences with regard to several endpoints, including weight (Chen and Kelly, 2005; Vaglenova et al., 2004). In particular, Chen and Kelly (2005) reported that females exposed to nicotine prenatally had higher body weights as adults. It is possible that this gender-specific outcome reflects differential long-term responses of male and female off-spring to altered hormonal levels (Sarasin et al., 2003; Lichtensteiger and Schlumpf, 1985). On the other hand, there might be gender-specific long-term consequences to nicotine-induced hypoxia and the subsequent release of stress hormones, which could set the stage for long-term weight-related changes. None of these would occur in this study because treatment is given postnatally. However, there was a non-significant trend towards a stronger affect on growth reduction in male pups exposed to nicotine, which was evident by the earlier onset of significantly decreased body weight (Fig. 2C, D) and a non-significant trend towards slightly increased body weight in nicotine-treated adolescent females (Fig. 4). Thus, it is possible that gender-specific effects are more pronounced in prenatal than postnatal animal models and in humans exposed to nicotine in utero.

In summary, we developed a postnatal animal model that allows the evaluation of nicotine during a developmental period that corresponds to the third trimester in humans. Using this model, the short-term and long-term effects of nicotine on postnatal growth were evaluated. We found a robust anorexic short-term effect of nicotine, with no apparent long-term consequences. This action appears to involve  $\alpha 4\beta 2$  nAChRs since it is blocked by DH $\beta$ E coadministration.

## Acknowledgements

This study was supported by the Department of Medical Pharmacology and Toxicology at TAMU-HSC and NIH Grant # DA016487, AA12386.

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