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Gender-related response in open-field activity following developmental nicotine exposure in rats

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Abstract

Smoking during pregnancy may lead to low birthweight and behavioral alterations in the offspring. In this study, the effects of developmental nicotine exposure on the somatic growth of the offspring and the behavioral performance in the open-field test were examined. Sprague–Dawley female rats were implanted with nicotine (35 mg for 21-day time release; NIC 35) or placebo pellets on gestational day (GD) 8 (postblastocyst implantation). A normal control group with no pellet implant was also included. There was a significantly higher maternal weight gain in the placebo group possibly due to a larger litter size. However, there were no significant differences in body weights among all three treatment groups for male and female offspring. The amount of activity, measured by the total number of crossings in the open-field test, indicated a gender difference in baseline level and pattern of ambulatory activity, with less activity (lower number of crossings) in male offspring and an increase in the activity of the female offspring as a function of testing day. The increase in the ambulatory activity of the female offspring was observed in the placebo and normal, but not the NIC 35 group suggesting that developmental nicotine exposure interferes with open-field activity, and this behavioral alteration is gender related.

Keywords: Behavior; Cigarette smoking; Pregnancy; Substance abuse

1. Introduction

Cigarette smoking is a socially acceptable and legal activity. However, it has a detrimental impact on smokers with respect to health issues such as addiction, lung cancers, and cardiovascular problems (Bartal, 2001). One of the most unfortunate facts about smoking is the double jeopardy of maternal smoking during pregnancy because it affects not only the health of the mother, but also the developing fetus. According to a report from the Centers for Disease Control and Prevention, it was estimated that 12.2% of all pregnancies in the United States alone involved tobacco smoking during the year 2000 (Martin et al., 2000). Among all of the toxic substances from cigarette smoking, nicotine's addictive property compels the smoker to continue smoking, although the Surgeon General's warning regarding cigarettes' harmful effects on pregnancy has appeared on cigarette packages since 1985.

Currently, the spectrum of the damaging effects of smoking prior to or during pregnancy includes decreases the success rate of becoming pregnant or carrying a baby to term (Economides and Braithwaite, 1994) and an increased number of births by cesarean section and a decrease of postpartum breastfeeding due in part to a lack of milk production (Habek et al., 2002; Leston et al., 2002). Moreover, smoking during pregnancy has been linked to an increased incidence of sudden infant death syndrome (DiFranza and Lew, 1995), a reduction in birthweight (Ernst et al., 2001), and disruptions in the functions of the central nervous system (Xu et al., 2001) on the affected offspring. Therefore, it is undisputed that smoking can interfere with normal pregnancy and the development of the fetus.

Unlike alcohol, smoking during pregnancy does not produce a constellation of distinctive abnormalities at birth. However, it is documented that some children exposed to maternal smoking during pregnancy exhibited alterations of

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specific behaviors. For example, affected children showed more episodes of fussiness and indifferent attitude while being held by an unfamiliar person (Kelmanson et al., 2002). Furthermore, a recent review by Wakschlag et al. (2002) summarized severe antisocial behaviors in offspring born to mothers who smoked during pregnancy. It is unclear whether nicotine or other ingredients within a cigarette are responsible for the behavioral alterations observed in children born to mothers who smoked during pregnancy. Frequently, the effects of maternal smoking are misinterpreted as the effects of nicotine, and the potential harmful effects of carbon monoxide, cyanide, or many other agents from cigarette smoking on the developing fetus are generally ignored. Therefore, it is important to determine whether nicotine is responsible for mediating some behavioral alterations observed in humans.

In this study, the role of nicotine, rather than other compounds in cigarettes was evaluated on performance in the open field. The open-field test is commonly used to evaluate locomotion, anxiety, and various alterations in emotional behavior (Fernandez et al., 1983, Lacroix et al., 2000; Oddie et al., 2002). For instance, McFadyn et al. (2002) tested the behavioral effects of methylphenidate (Ritalin), the widely prescribed medication for attention deficit hyperactivity disorder, using the open-field test. Similarly, Tzavara et al. (2002) applied the same test to study behavioral distress following nicotine withdrawal. In this study, the open-field task was used to test the hypothesis that neonatal rat pups that were exposed to nicotine during brain development would display deviations in their behaviors assessed during adulthood compared with control animals suggesting that nicotine is a behavioral teratogen. In addition to the behavioral assessment, the somatic growth of the offspring was monitored, along with other important parameters, such as maternal weight gain, litter size, and gender ratio following developmental nicotine exposure.

2. Methods

2.1. Subjects

Nineteen pregnant adult female Sprague–Dawley rats (~ 220 g in weight at the commencement of the experiment) received nicotine or control treatment, and 33 offspring (15 male and 18 female) from these treated dams were used in the open-field study. No more than one male or/and one female pup from the same treated dam was/were used in the behavioral testing. The adult female rats were obtained from Harlan (Houston, TX), and their offspring were born and reared in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care)-accredited facility at The Texas A&M University System Health Science Center. The colony was maintained at 23 °C, with a standard 12-h light/12-h dark photoperiod (light on at 6:00 a.m.). The food (rat chow) and water was available to the animals, both dams and offspring, throughout the experiment. The experimental procedures used in this study were approved by the University Laboratory Animal Care Committee at Texas A&M University.

2.2. Breeding and treatment procedures

The offspring of nicotine- or control-treated Sprague–Dawley rats served as subjects in this study. For the breeding process, each virgin female rat was housed overnight with a proven male breeder rat. The following morning, a sample taken from the vaginal canal was examined for the presence of sperm. The day that the sperm was identified in the vaginal smear was denoted as gestational day (GD) 0. On GD 8, each pregnant female rat was assigned to one of three treatment groups: NIC 35 (35 mg nicotine pellet), placebo (0 mg nicotine pellet), or normal (no nicotine pellet implanted). Subjects assigned to the NIC 35 and placebo groups were implanted with either a 35-mg (21-day timed release) or 0-mg nicotine pellet (Innovative Research of America, Sarasota, FL), respectively. The normal group received no pellet implantation to control for the potential effects of the implantation procedure performed during midgestation (GD 8). The rationale for the selection of GD 8 (following blastocyst implantation) was based on our previous finding showing that the 35-mg nicotine pellet given on GD 0 (day of conception) failed to produce viable fetuses in four of five dams that were identified as sperm positive, and no implantation sites were identified in the uterine horns of these dams by visual inspection or 10% ammonium sulfide immersion on GD 20 (Chen and Eskue, 2002). In addition, the choice of the 35-mg nicotine pellet was based on the consideration that (1) 15 or 25 mg nicotine pellets given from GDs 0–21 did not produce significant and obvious teratogenic effects either in various developmental parameters or neural development as measured by neuronal loss (Chen and Edwards, 2003) and (2) this nicotine dosing regimen would be equivalent to 6.5 mg/kg/day (on GD 8) to 4.4 mg/kg/day (on GD 21), which were similar to the dosage reported in the literature (Fewell et al., 2001a; Slotkin et al., 1997; Xu et al., 2001). The blood nicotine level was not measured in this study. Slawecki and Ehlers (2002) showed that 5 mg/kg/day nicotine administered to the adolescent rats via transdermal Nicoderm CQ patches resulted in a mean blood nicotine level at 88.5 ng/ml. Furthermore, Slotkin et al. (1997) and Xu et al. (2001) reported that administration of 6 mg/kg/day nicotine (via osmotic minipump) to rats produced a higher blood nicotine level compared with the levels in average smokers. However, it should be noted that the extrapolation of blood nicotine concentration between humans and animals should be cautiously conducted, because of various factors, such as smoking habits and the brand of cigarettes smoked.

For the implantation procedure, animals were anesthetized with methoxyflurane (Metofane®, Schering-Plough Animal Health, Union, NJ), and either the 35-mg nicotine

or placebo pellets were implanted in the dorsal aspect of the neck using aseptic surgical procedures. The dorsal incision was closed with stainless steel wound clips, and the subjects were allowed to recover fully from anesthesia before being returned to the vivarium. One important fact that requires emphasis is that the pups of the NIC 35 groups were exposed to nicotine possibly up to postnatal day (PD) 7 through the mother's milk (American Academy of Pediatrics Committee on Drugs, 2001; Perlman et al., 1942), since the nicotine pellet implanted (to the dam) was designed for 21-day release. Therefore, the current exposure paradigm was termed "developmental nicotine exposure" rather than "prenatal nicotine exposure," since the period of exposure potentially encompassed part of the third trimester equivalent period (the brain growth spurt) in rats.

2.3. Maternal and offspring variables

Starting on GD 0, each pregnant rat was singly housed, and food and water was available ad libitum. The body weight of these pregnant females was monitored daily until GD 20. The day that pregnant rats gave birth was denoted as PD 0. The pups and dam were left undisturbed on PD 0. On PD 1, the number of male and female viable offspring was counted, and each litter was randomly culled to five male and five female pups when possible. The mean body weight on PD 1 from a litter was measured from all pups of the same gender prior to the culling procedure. The culled pups were raised by their biological mothers and weaned on PD 21, after which they were housed two to three (same gender) per cage until PD 35 and two per cage beyond PD 35. Body weights were recorded daily until weaning, then recorded weekly until PD 56. Some body weight data were unavailable beyond PD 35; therefore, the mean litter body weight was reported only up to PD 35. In addition to the body weight data of the dams and the offspring, other parameters, such as litter size and gender ratio, were also measured.

2.4. Open-field behavior test

On PD 60, the experimental subjects from all treatment groups were subjected to open-field behavior testing conducted between 2:00 p.m. and 4:00 p.m. daily for three consecutive days. The experimental subjects were one male and one female, when possible, from a treated dam. In other words, no more than one offspring from the same gender would be subjected to the open-field test. Each daily test session consisted of two 2-min trials, with an intertrial interval of 1 min. The open-field apparatus was a $91.4 \times 91.4 \times 25.4$-cm $(36 \times 36 \times 10$ in.) $(L \times W \times H)$ wooden box painted gray with a solid floor demarcated into thirty-six 15.2-cm (6-in.) squares. Prior to each testing trial, the internal surface of the apparatus was cleaned with Nolvasan (1:10 ratio of Nolvasan to water) to eliminate any olfactory cues from the previous trial. The spatial contextual cues remained constant throughout the experiment. At the beginning of each trial, the subject was placed in a corner square of the open-field apparatus (locations of placement were different between the two daily trials) and the subject's behavior was videotaped for the duration of the trial. The total number of line crossings for each trial was scored (a crossing was defined as the entry of two forepaws from one square to an adjacent square), and the mean

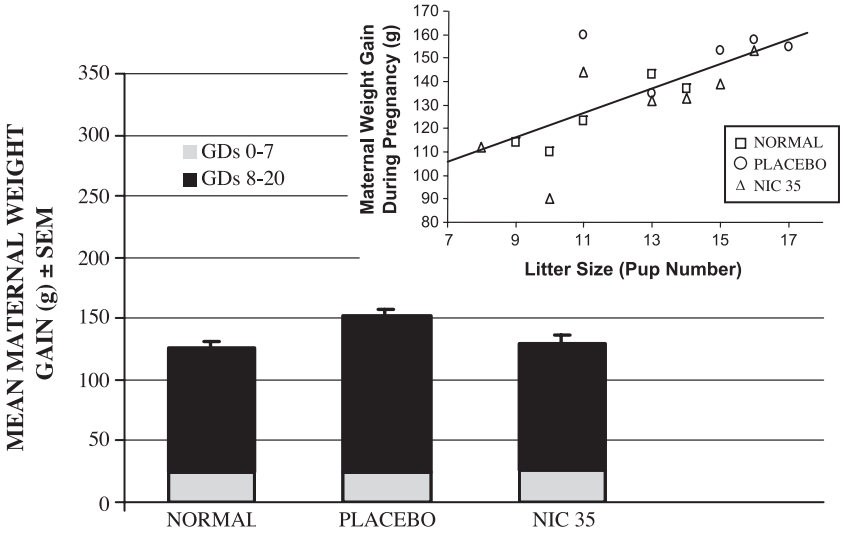

Fig. 1. The mean maternal weight gain as a function of nicotine treatment. The weight gain between conception and pellet implantation was denoted as GDs 0–7, and the weight gain following the pellet implantation was denoted as GDs 8–20. There was no difference for the weight gain between GD 0 and GD 7. However, the weight gain between GD 8 and GD 20 was significantly different among all three groups [$F(2,14) = 6.62$, $P < .05$]. The n for the maternal weight analysis was 7, 5, and 5 for NIC 35, placebo, and normal, respectively (the weight for two normal dams on GD 0 were unavailable, therefore, five datum points were used in analyzing maternal weight gain). The insert regression scatter plot demonstrated the relationship between maternal weight gain and litter size (the regression line of Y on X: $Y = 5.38X + 66.4$). The vertical bars represent the S.E.M.

Table 1
The mean ± S.E.M. offspring body weight (g) at three developmental landmarks as a function of nicotine treatment

	Male			Female		
	PD 1	PD 21	PD 35	PD 1	PD 21	PD 35
Normal ($n=7$)	7.4 ± 0.1	53.1 ± 1.1	140.6 ± 2.5	7.2 ± 0.2	51.2 ± 1.0	119.7 ± 1.9
Placebo ($n=5$)	7.3 ± 0.2	53.9 ± 2.4	142.8 ± 4.8	7.3 ± 0.3	53.0 ± 0.9	123.4 ± 2.7
NIC 35 ($n=7$)	7.3 ± 0.3	52.2 ± 1.7	139.7 ± 2.5	6.9 ± 0.2	50.7 ± 1.6	120.3 ± 2.4

number of crossings for the two daily trials represented the crossing for each testing day.

3. Results

3.1. Maternal weight gain and litter size

The analysis of variance (ANOVA), with treatment as the between factor, conducted on the maternal weight gain data between GD 0 and GD 7 showed no significant difference among all three groups. However, the maternal gain from GD 8 to GD 20 (after nicotine/placebo pellet implantation) showed a significant main effect of treatment [$F(2,14)=6.62$, $P<.05$]. Fisher's LSD (least significant difference) post hoc tests showed a significantly higher weight gain in the placebo group than that in the NIC 35 and normal groups (Fig. 1). The higher weight gain in the placebo group may be due to the larger litter size with 14.4 ± 1.1 pups per litter compared with 12.4 ± 1.1 and 12.4 ± 0.9 pups per litter for the NIC 35 and normal groups, respectively (Fig. 1, inset). However, there was no statistically significant difference in litter size suggesting that developmental nicotine treatment did not significantly affect the litter size. With regard to the male to female offspring ratio, no significant differences were found among three treatment groups. The mean male to female ratios for the NIC 35, placebo, and normal groups were 1.11 ± 0.42, 0.84 ± 0.18, and 0.80 ± 0.13, respectively. From evaluating the standard errors of the mean (S.E.M.), it appeared that the variability was greater in the NIC 35 group compared with those in the placebo and normal groups, since there were three litters having only two male pups and one litter having three female pups in the NIC 35 group.

3.2. Offspring somatic growth

The mixed ANOVA, with treatment and gender as between factors and PD (PDs 1, 21, and 35) as a within (repeated) factor, performed on offspring body weight data showed no main effect of treatment, that is, the developmental nicotine treatment starting on GD 8 did not interfere with the postnatal offspring growth on PDs 1, 21, and 35. However, there were significant differences in gender [$F(1,32)=38.58$, $P<.01$] and PD [$F(2,64)=11{,}456.97$, $P<.01$]. Furthermore, there was a significant gender and PD interaction [$F(2,64)=88.99$, $P<.01$]. The locus of the interaction was due to a significantly higher body weight in male than in female offspring on PD 35. However, the weights on PD 1 or 21 were not different between male and female offspring (Table 1).

3.3. Open-field activity

Three 2-way ANOVAs were performed with gender and treatment as the two between factors on three testing days. These ANOVAs showed a significant main effect of GENDER on all three testing days [$F(1,27)=4.23$, 24.58, and 21.23, Ps $<.05$ for Days 1, 2, and 3, respectively]. Neither a main effect of treatment nor a significant interaction between

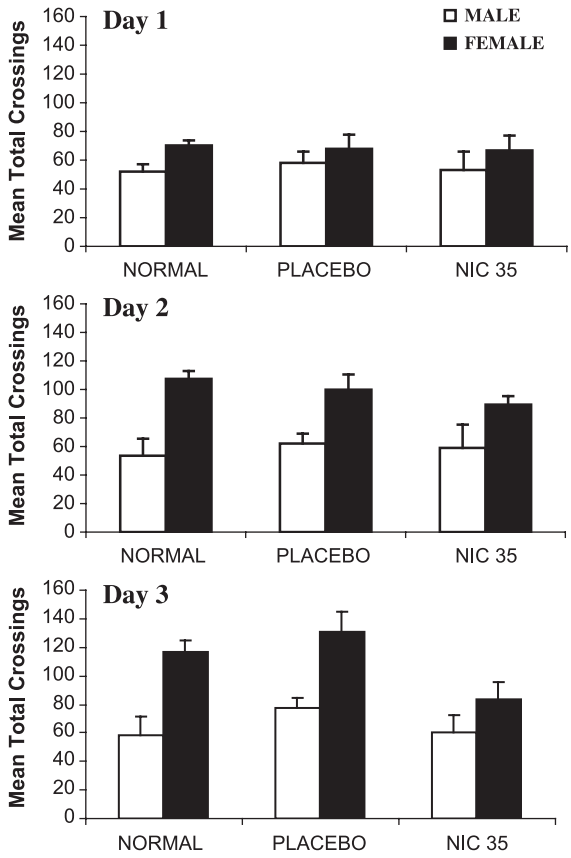

Fig. 2. The mean total crossings in the open-field test as a function of nicotine treatment and gender. Due to the variability in the numbers of female and male offspring born, there were six, five, and seven female offspring, and four, four, and seven male offspring available for this open-field testing for the NIC 35, placebo, and normal groups, respectively. The ANOVA for each testing day showed a significant gender effect [$F(1,27)=4.23$, 24.58, and 21.23, Ps $<.05$ for Days 1, 2, and 3, respectively]. The vertical bars represent the S.E.M.

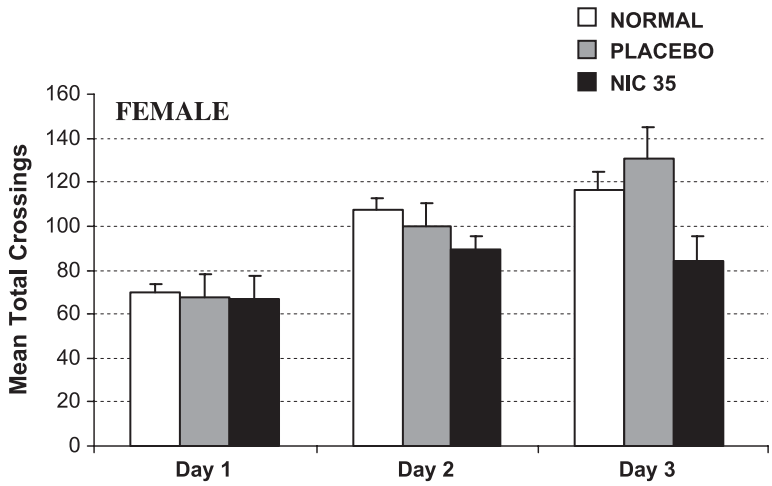

Fig. 3. The mean total crossings in the open-field test as a function of nicotine treatment and testing day in female rats. Separate ANOVAs using treatment and testing day as independent variables showed a main effect of testing [$F(2,30)=38.33$, $P<.05$] and an interaction between testing day and treatment [$F(4,30)=3.90$, $P<.05$] in female, but not in male, rats. Further analyses on female data demonstrated that the total number of crossing was lowered in the NIC 35 group compared with those in the placebo and normal control groups only in the third day of testing. The vertical bars represent the S.E.M.

gender and treatment was revealed (Fig. 2). Since there is an inherited difference between male and female rats in this open-field test, further analyses were conducted without gender as a between factor to further examine the activity as a function of testing day. Two mixed ANOVAs, with treatment as a between factor and testing day as a within (repeated) factor, were conducted on the total crossing data. In female offspring, the analysis showed a significant effect of testing day [$F(2,30)=38.33$, $P<.05$] and an interaction between treatment and testing day [$F(4,30)=3.90$, $P<.05$]. With respect to the interaction, further one-way ANOVAs revealed that the locus of the interaction was on the third day of testing [$F(2,15)=4.56$, $P<.05$] with significantly higher numbers of crossing in the placebo and normal groups, but not in the NIC 35 group (Fisher's PLSD post hoc test) (Fig. 3). However, in male offspring, there was no significant main effect of treatment, testing day, or an interaction between these two factors.

4. Discussion

The mean body weight of the male and female offspring was not significantly different among the three treatment groups as measured on PDs 1 (day after birth), 21 (day of weaning), and 35 (adolescent period). These findings were similar to those reported by Sobrian et al. (1995) suggesting that prenatal nicotine has a limited effect on somatic growth as measured by body weight. The finding of no difference on PD 1 weight between nicotine and control treatments was not consistent with the general view in terms of maternal smoking and low birthweight. Although the weight of PD 1 pups was not the birthweight, our data indicated no weight deficits just 1 day after birth. Therefore, even if the birthweight of nicotine-treated pups was lower than those of controls, such a deficit was transient since normal somatic growth was observed during the developmental periods in nicotine exposed offspring. Similarly, this finding did not coincide with some clinical reports indicating that the body weights of young children born to mothers who smoked during pregnancy were actually higher than those of children born to nonsmokers (Von Kries et al., 2002). Although it is still unclear whether such an increase in body weight was a function of environmental factors postnatally or exposure to many toxic compounds from cigarette smoking prenatally. The lack of a nicotine-induced change in body weight from the current and other studies (Newman et al., 1999) suggests that compounds other than nicotine from cigarette smoking, such as carbon monoxide or cyanide (which affects thyroid status), may play a role in altering somatic growth.

The open-field data from this study showed a gender difference with a significantly lowered mean number of crossings in male rats compared with those of female offspring in all three days of testing. This gender difference in the overall activity (total number of crossings) was not a novel finding (Brotto et al., 2000; Gray and Cooney, 1982; Meng and Drugan, 1993; Slob et al., 1986). It is a consensus that female rats are less anxious than male rats in an open-field environment, thereby exhibiting more ambulatory activity (for reviews, see Kelly et al., 1999; Palanza, 2001).

Upon further evaluation of the data from the perspective of testing days, the male and female data showed a different pattern in open-field activity. The female offspring in both placebo and normal control groups, but not in the NIC 35 group, showed a gradual increase in the ambulatory activity on the second and third days of testing. This finding suggested that the control subjects in the placebo and normal groups adapted to or were able to learn/retrieve the information about the testing apparatus, thereby showing

much less anxiety or fear in this environmental context, thus, the ambulatory activity increased as a function of testing day. Following the same line of interpretation, the female offspring from the NIC 35 group did not show a gradual increase in the activity on the second and third testing days, suggesting a deficit of these animals to adapt to the open-field context, to learn the contextual cues, or to retrieve the learned information regarding the familiarity of the open-field environment. This interpretation is different from the view that female rats generally habituate to the "nonnovel" context, thus leading to a decrease in ambulatory activity (Tropp and Markus, 2001). It is speculated that the duration of each testing episode (trial) and the history of handling may account for the discrepancy. Meng and Drugan (1993) demonstrated that a gender difference was observed in the open-field activity with a 5-min testing episode, however, such a gender difference disappeared with a 7.5-min testing episode suggesting the female rats are more active in the early phase of the testing and the activity decreases during a late phase of a long testing episode. In other words, as the testing duration prolonged, the female rats may exhibit habituation to the context, thus decreasing the ambulatory activity. Furthermore, an earlier paper by Denenberg (1969) showed that handled rats exhibited increased activity as a function of testing days while the nonhandled rats showed a decrease in activity. Since the subjects used in the current study were handled frequently, particularly during the early stage of development (before weaning), such handling experience may contribute to the increase in the activity observed in the female offspring on the second and third days of testing.

For male offspring, there were no changes in terms of the total crossings across all three testing days regardless of developmental nicotine treatment. These data might be a result of ceiling/floor effect in their activity under this particular testing regimen for male rats. However, the absence of difference in total crossings between the NIC 35 and control subjects needs to be cautiously interpreted. Although the baseline activities are similar, the offspring in the NIC 35 group may respond differently to pharmacological challenges or other stimulations due to developmental nicotine exposure. Similar phenomena have been reported previously in various experimental paradigms involving prenatal exposure to alcohol, cocaine, or nicotine (Ankargberg et al., 2001; Chen et al., 1997; Nagahara and Handa, 1999). It is likely that if an acute pharmacological challenge or other stimulations were given to the developmental nicotine-treated male offspring, the responses to that challenge or stimulation could be different from those receiving no nicotine during development.

It is worth noting that each animal model has its advantages and limitations. The current nicotine exposure method delivered a constant, steady amount of nicotine to the pregnant animals similar to the osmotic minipump. This "nicotine patch-like" administration route has been considered an optimal route of administration in many laboratories studying prenatal nicotine effects on offspring (Fewell et al., 2001b; Popke et al., 1997; Sobrian et al., 1995; Xu et al., 2001). Nevertheless, this route of administration does not closely mimic the clinical condition (smoking) that generally results in spikes of peak nicotine level in the physiological system. The discussion and comparison of relevant routes of drug administration is definitely beyond the scope of this study, yet it is important to recognize limitations since it may contribute significantly to the interpretation of experimental outcome.

In summary, "nicotine patch-like" nicotine exposure following blastocyst implantation did not result in somatic growth retardation in the resultant offspring, nor did it affect the number of offspring born to the treated dam. However, such a developmental nicotine treatment altered open-field performance in female adult offspring, but not in the male offspring, suggesting that gender is a relevant and significant factor in the assessment of developmental nicotine exposure.

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