Available online at www.sciencedirect.com
ScienceDirect
Pharmacology, Biochemistry and Behavior 85 (2006) 835–841
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
www.elsevier.com/locate/pharmbiochembeh

Long-term behavioral and developmental consequences of pre- and perinatal nicotine

Amy K. Eppolito *, Robert F. Smith

George Mason University, MS 3F5, 4400 University Drive, Fairfax, Virginia 22030, United States

Received 21 February 2006; received in revised form 20 November 2006; accepted 21 November 2006
Available online 28 December 2006

Abstract

Research has shown that cigarette use during pregnancy can result in increased fetal mortality, sudden infant death syndrome, and behavioral and attentional disorders during childhood. Neurochemical and behavioral consequences of prenatal nicotine exposure have been well documented although few studies have examined long-term behavioral consequences that persist into adulthood. In this study, fifty-eight male and female Long–Evans rats were exposed to chronic nicotine prenatally and postnatally via subcutaneous infusions (0.96 mg/kg/day) in the dam. Nicotine exposure continued in the pups via maternal milk until the dams' osmotic mini-pumps became exhausted at approximately postnatal day (P) 11. At weaning, animals were group housed until behavioral testing at P60 to assess spatial learning and memory in the Morris water maze (MWM). Mild deficits in spatial learning were observed in nicotine-exposed females. These behavioral differences were accompanied by significant reduction in weight gain of nicotine-exposed females beginning at puberty, suggesting a hormonal interaction. Long-term effects of nicotine exposure were less striking in males. Nicotine-exposed males had significantly slower swim speeds than controls, but latency to reach the hidden platform was equal between groups by the conclusion of testing. Weight gain in males did not differ between groups as a result of prenatal nicotine exposure.

Keywords: Nicotine; Prenatal; Morris water maze; Spatial learning; Puberty; Growth

1. Introduction

Human and animal research has demonstrated multiple teratogenic effects of drug use on development during gestation. Despite this, many women continue to use drugs and alcohol during pregnancy. One of the most commonly abused drugs is nicotine. In fact, an alarming 11% of pregnant women in the United States report smoking at some point during, or throughout their pregnancies (Martin et al., 2005). Cigarette use during pregnancy has been correlated with spontaneous abortion, increased fetal mortality, sudden infant death syndrome, decreased IQ scores, behavioral disorders such as hyperactivity and conduct disorder (Ernst et al., 2001), and deficits in fine motor skills, attention, and auditory processing (Cutler et al., 1996). Epidemiological studies have also linked cognitive deficits to prenatal cigarette exposure. Deficits in neuropsychological development on tasks that require learning, memory, and problem solving skills were impaired in children up to age 10 following exposure to maternal smoking during gestation (Cornelius et al., 2001; DiFranza et al., 2004). Additional studies have shown links between maternal smoking and behavior disorders including attention deficit hyperactivity disorder (ADHD) (DiFranza et al., 2004). Nicotine is a known neuroteratogen, which halts neuronal cell replication, leading to a decrease in total cell count, and subsequent deficits in synaptic connectivity and neurochemical activity (Xu et al., 2001).

Numerous animal studies have shown that the neurobehavioral and cognitive effects associated with prenatal nicotine result in deficits similar to those seen in humans, including increased psychomotor activity, cognitive impairments, and low birth weight (Levin et al., 1993). Attentional and spatial memory deficits have been noted in the Morris water maze (MWM) and radial-arm maze tasks in prepubertal, adolescent, and adult rats prenatally exposed to nicotine (Cutler et al., 1996; Levin et al., 1993; Sorenson et al., 1991). Further evidence suggests that cognitive changes are a result of long-lasting alterations to the

* Corresponding author. Tel.: +1 703 993 1358; fax: +1 703 993 1359.
E-mail addresses: aeppolit@gmu.edu (A.K. Eppolito), bsmith@gmu.edu (R.F. Smith).

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doi:10.1016/j.pbb.2006.11.020

nicotinic and adrenergic neurotransmitter systems following in utero exposure to nicotine (Levin et al., 1993).

Neurochemical changes including the disruption of neuronal migration, cell proliferation, and cell differentiation along with altered functionality of the cholinergic and catecholaminergic neurotransmitter systems have been observed in rats exposed to prenatal nicotine (Ernst et al., 2001). Chronic exposure to nicotine during prenatal development results in an upregulation and increased receptor binding of dopamine (DA) and norepinephrine (NE) suggesting that nicotine slows synaptic activity (Levin et al., 1993). These neurochemical changes likely explain some of the behavioral deficits noted in animals exposed to nicotine prenatally.

Differences in developmental time course between species must be considered if animal research is to be generalized to humans. Rat gestation is typically 21 days, but the rat pup does not reach a level of cerebral maturity equal to the human at birth until P10. The third trimester equivalent in the rat occurs postnatally at approximately P4–P9 (Dobbing and Sands, 1979). In order to examine the effects of nicotine into the period of development equal to the third trimester in humans, it is critical to continue dosing the pups from P1–P10. Most studies have ended prenatal dosing just before birth, ignoring this important period (Cutler et al., 1996; Levin et al., 1993, 1996; Roy et al., 2002; Shacka and Robinson, 1998; Xu et al., 2001).

An important consideration when using animal models for nicotine research is mimicking the relatively steady-state plasma concentrations of nicotine that human smokers achieve. Injections of nicotine result in episodic hypoxia and spikes in nicotine plasma concentrations, potentially confounding any drug affects that might occur (Slotkin, 1998). Oral administration of nicotine has been found to reduce feeding behaviors in pregnant dams, preventing a steady-state plasma concentration of nicotine (Murrin et al., 1987). To date, the best mechanism for delivering nicotine at a relatively constant rate is the osmotic mini-pump. Analysis of nicotine serum levels in the rat following use of the osmotic mini-pump have shown consistent levels from animal to animal (Lichtensteiger et al., 1988; Murrin et al., 1987). However, this method of dosing does not take into account daily fluctuations in nicotine serum levels seen in smokers, who maintain relatively constant levels of plasma nicotine concentrations throughout waking hours with a gradual decline overnight, followed by increasing levels through the morning hours. These daily alterations in plasma nicotine concentrations may afford some protection to the developing human fetus (Slotkin, 1998). Constant infusion of nicotine also makes it difficult to identify critical periods of prenatal development during which nicotine may be more damaging (Ernst et al., 2001). Despite these caveats, the osmotic mini-pump is a more ecologically valid method of nicotine administration, and reduces stress to the dam that result from oral and subcutaneous methods of administration.

Use of the 28-day osmotic mini-pump provides an elegant design for continued exposure to the young rat pup through maternal milk. Nicotine is excreted in breast milk, providing a methodology for perinatal exposure to the rat pup, while minimizing stress associated with other methods of dosing (e.g. injections, oral gavage). There is evidence in rats that nicotine exposure via maternal milk alters nicotinic acetylcholine receptor (nAChR) expression in perinatal pups (Narayanan et al., 2002). Specifically, a significant dose-dependent upregulation of cortical nicotinic receptors was found in rat pups exposed to nicotine at 2, 4, and 6 mg/kg/day through maternal milk on P3–P18. While the exact levels of nicotine excreted through breast milk to the pups are unknown, the level was sufficient to induce upregulation of nAChRs, with the potential to alter long-term the functioning of synaptic activity and neuronal development (Narayanan et al., 2002).

It is important to determine critical periods of prenatal development that result in adverse behavioral and neurochemical consequences following nicotine exposure. For this reason particularly, dosing past birth in the rat, during a developmental period that correlates with the third trimester of human pregnancy, is important. Here, we examine the long-term effects of prenatal and early postnatal nicotine exposure on cognitive performance in the MWM.

2. Method

Ten virgin Long–Evans female rats (Harlan, Indianapolis, IN) were housed with males until detection of a sperm plug, which was designated as GD0. Sperm positive females were removed from the breeding cages and housed individually in nesting boxes with ad libitum food and water. A total of 58 male (control: $n=14$; nicotine: $n=17$) and female (control: $n=14$; nicotine: $n=13$) offspring were used in the study. A 12-hour light/dark cycle (lights on at 7:00AM) was maintained in standard laboratory conditions, temperature 20–23 °C, and humidity 40–60% throughout the study. All animal care and experimental protocols were in full compliance with George Mason University guidelines set by the Institutional Care and Review Committee for the Use of Animal Subjects and the National Institutes of Health Guide for Care and Use of Laboratory Animals (Institute for Laboratory Animal Resources, 1996).

At GD4 sperm positive dams were randomly assigned to either nicotine or saline control drug groups, anesthetized with equithesin (3 mg/kg) (Sinnayah et al., 1999) and implanted with a 28-day Alzet osmotic mini-pump (Model 2004, Durect Corporation, Cupertino, CA). GD4 was used as the date of pump insertion because embryos are not yet implanted in the wall of the uterus, thus minimizing the potentially harmful effects of anesthesia on the developing embryos (Levin et al., 1996; Roy et al., 2002). To prevent interruptions in maternal care, the osmotic mini-pumps were not removed from the dams until after the pups were weaned.

Pumps were filled with a solution of nicotine (nicotine hydrogen tartrate salt, Sigma, St. Louis, MO) sufficient to release an initial dose of 0.96 mg/kg/day (freebase), or with 0.9% normal saline (NaCl) and allowed to incubate at 37 °C for 48 h prior to implantation. This incubation period allows for immediate delivery of the solution on implantation. With a flow rate of 0.22 μl/hour, and a fill volume of 237 μl, pumps were exhausted in roughly 28 days, ensuring nicotine exposure to pups via maternal lactation to P11 (Narayanan et al., 2002).

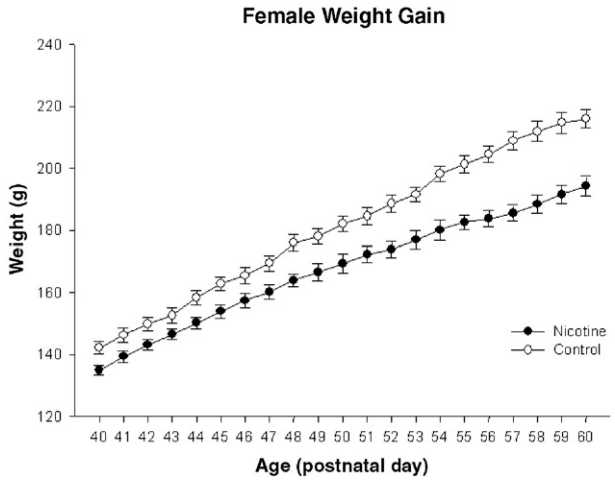

Fig. 1. Weight gain in females from P23–P60. There was a significant deficit in weight gain among females exposed to nicotine prenatally ($p<0.05$), beginning at P45 and persisting into adulthood.

Exposure to nicotine during the early prenatal period in the rat is critical in order to mimic exposure that would occur in the third trimester of human development among women who smoke during pregnancy (Dobbing and Sands, 1979). The relatively low dose rate was used to correlate with smoking half a pack or less per day. Women who report tobacco use during pregnancy report smoking 1–10 cigarettes per day (Martin et al., 2005). Blood plasma analysis indicates that in the rat, 1.5–2.0 mg/kg/day is equivalent to smoking one pack of cigarettes per day (Ernst et al., 2001; Levin et al., 1993; Murrin et al., 1987). In the rat, infusions at a rate of 2 mg/kg/day from GD4–GD20 do not affect maternal weight gain or lead to an increase in resorption of litters (Navarro et al., 1989).

Maternal weight gain was recorded daily until GD20. One nicotine dam and 2 control dams did not deliver litters. Three control dams and 4 nicotine-exposed dams delivered litters following 21–22 days of gestation. Pups were sexed and weighed within 24 h of parturition, and litters were culled to 10 pups, keeping 5 males and 5 females per dam whenever possible. Four pups were fostered to other dams in the same treatment condition to keep litter size relatively constant and control for nutritional confounds. There were no known cases of early postnatal mortality in any of the litters.

Pups were identified by ear punch at weaning (P22), and were housed by sex and drug condition in an enriched group-housing environment consisting of four three-level wire mesh cages, containing plastic chew toys and running wheels. Standard laboratory rat chow (Harlan Teklad Diet 7012) and water were available ad libitum throughout the duration of the study. Pups were weighed daily throughout the duration of the study.

The MWM was used to assess spatial learning beginning on P60, an age that is considered early adulthood in the rat (Spear, 2000). The pool measured 1.76 meters in diameter and was lined with a white polyurethane sleeve. White nontoxic tempera paint was added to the pool to make the water opaque and allow the computer controlled imaging system (HVS Image, LTD; Buckingham, UK) to track the black head of the rat. The escape platform was submerged 2 cm below the surface of the water and remained in the same quadrant of the pool for the duration of testing. Large black extra-maze cues were hung on the white curtains that surrounded the pool. Water temperature was maintained at 22–25 °C throughout testing. Ambient noise was kept to a minimum during testing.

Morris water maze testing spanned 5 days, with 3 trials per day, referred to as A, B, and probe trials. The trials were 60 s each, with start positions randomly determined from each quadrant of the pool. At the start of each trial, each rat was placed in the water with its head facing the wall of the pool. If the rat failed to find the platform within 60 s, it was directed to the platform location by the experimenter's hand, and allowed to sit for 15 s. Each time the rat located the platform before the trial end, it was allowed to sit there for 10 s before returning to the cage. Each trial was followed by a 30-second interval resting in the cage.

A modified Atlantis platform protocol was used. Trials using the Atlantis platform were called probe trials. Starting on day 2 of testing, the platform was lowered to the bottom of the pool

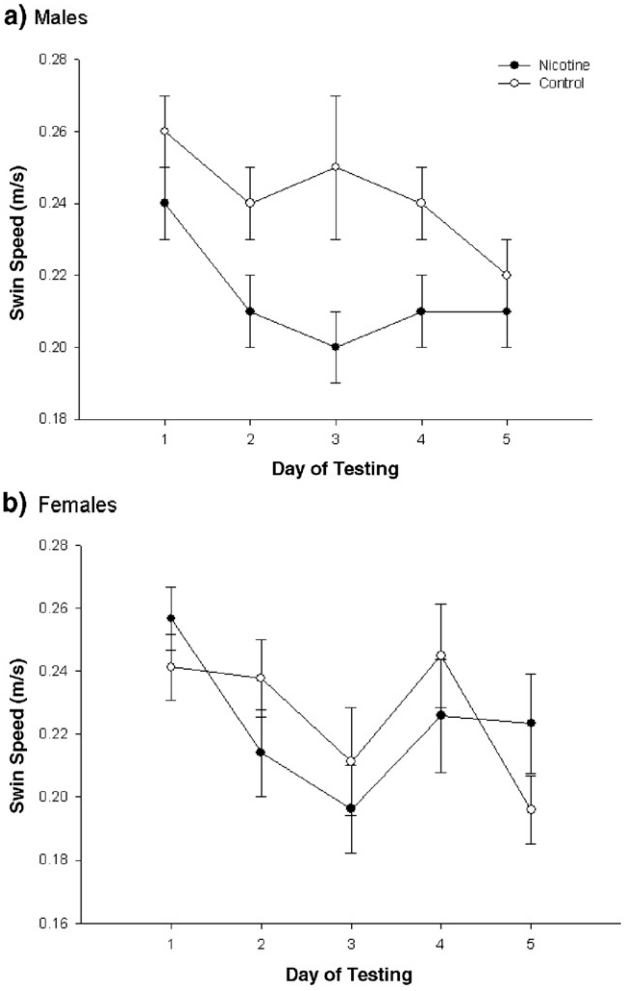

Fig. 2. Mean swim speed in meters/second, A and B trials blocked. a) A repeated measures nested ANOVA revealed a significant effect of prenatal nicotine-exposed males had significantly slower on swim speed in male rats in the MWM ($p<0.05$). When collapsed across all days of testing, the nicotine-exposed males had significantly slower swim speeds than controls. b) There were no significant differences in swim speed between groups in female rats.

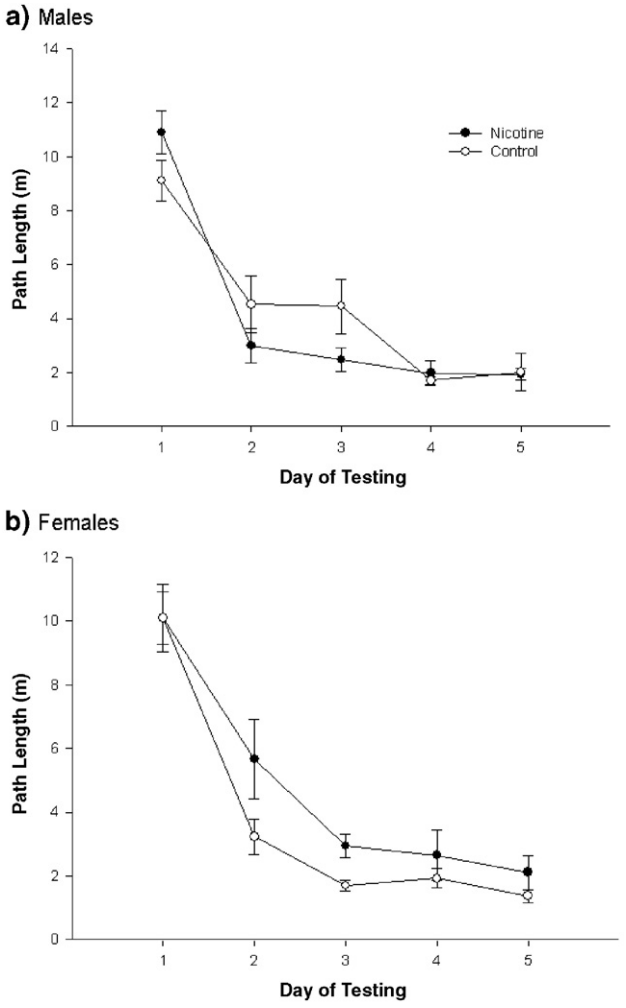

Fig. 3. Mean path length in meters, A and B trials blocked a) There were no significant differences in path length between groups in male rats. b) When collapsed across all five days of testing, repeated measures nested ANOVA revealed significantly longer path lengths in nicotine-exposed female rats ($p<0.05$).

every third trial, preventing the rat from escaping the water. The collapsible platform was modeled after the description by Buresova et al. (1985), and was controlled by series of pulleys and a fishing line that exited through the side of the pool. This set-up allowed the experimenter to pull the platform up at the end of each trial, and the rat escaped to the platform without seeing the experimenter. Before the start of each probe trial, the platform was simply pushed to the bottom of the pool. Each of these trials was 60-seconds, and when the trial ended, the platform was immediately raised and the rat had an additional 30 s to locate the platform. If the rat located the platform within the final 30 s, it was allowed 10 s resting on the platform before being removed from the pool, and returned to the cage. If the rat did not find the platform during the last 30 s, it was directed to the platform by the experimenter's hand, and allowed to sit on the platform for 15 s before returning to the cage.

The Atlantis water maze paradigm is considered a better test of spatial learning because it requires that the rat learn the exact location of the platform relative to visual extra-maze cues rather than rely on search strategies such as swimming in concentric circles until bumping into the platform. It is also a more stringent test of spatial learning and memory than the stationary platform used in the traditional MWM (Buresova et al., 1985; Spooner et al., 1994).

Repeated measures ANOVA was used to analyze daily maternal weight gain during gestation, and postnatal weight gain from birth to P60 in offspring. Analyses of litter size at parturition and pup birth weight were determined by separate one-way ANOVAs.

For analysis of behavior in the MWM, we employed a nested ANOVA for repeated measures with individual pups nested within litter; dose condition was the independent factor. Nested designs employ an additional error term and adjusted degrees of freedom that account for variability due to lack of independence (Keppel, 1991). The nested ANOVA controls for potential effects of litter membership.

Because previous research has indicated sex effects following exposure to nicotine during development, (Levin et al., 1993; Lichtensteiger et al., 1988; Xu et al., 2001; Sorenson et al., 1991)

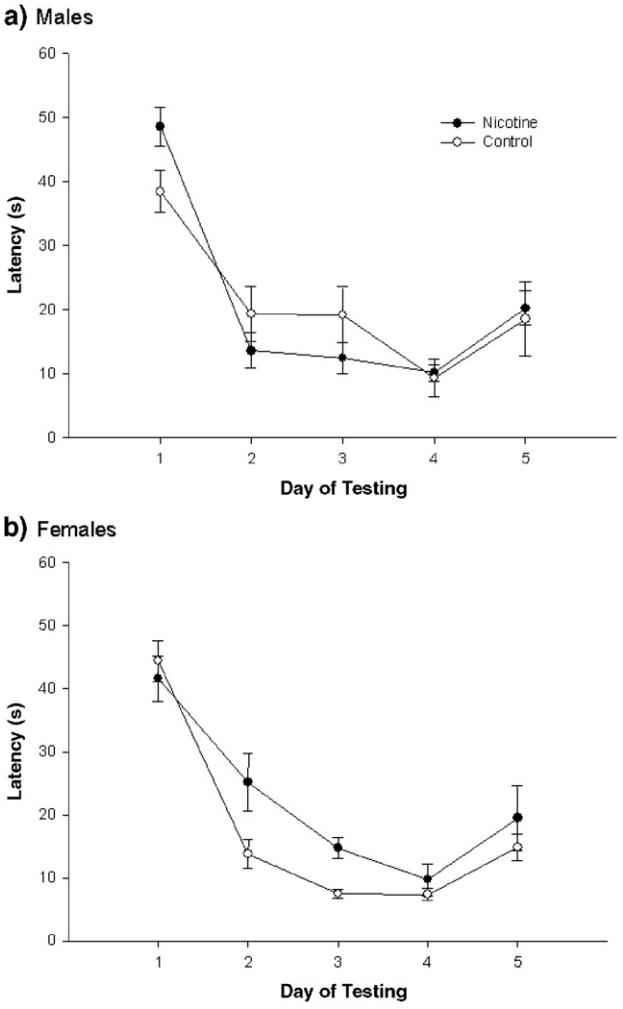

Fig. 4. Mean latency in seconds, A and B trials blocked. a) There was no overall effect of prenatal nicotine exposure on latency to reach the hidden platform in males. b) A repeated measures nested ANOVA revealed a significant effect of prenatal nicotine exposure on latency to reach the hidden platform in females ($p<0.05$) with nicotine-exposed females taking longer to locate the hidden platform.

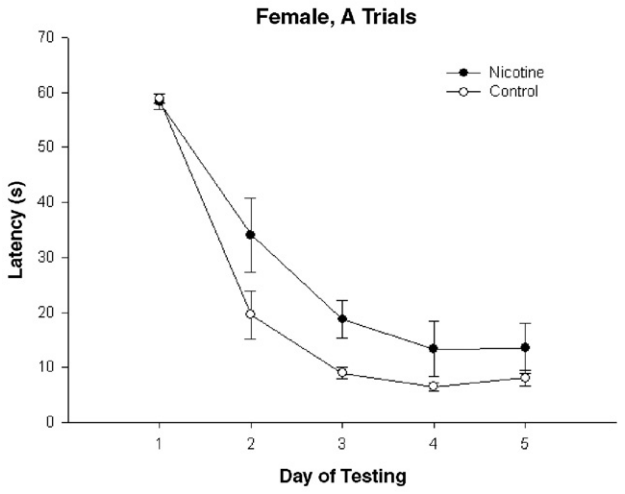

Fig. 5. A trials females, mean latency in seconds. The longer latencies in nicotine-exposed females indicated a deficit in reference memory. A trials indicate a deficit in memory for the location of the hidden platform ($p<0.05$).

these data were analyzed separately by sex. Dependent behavioral measures included latency to reach the hidden platform, thigmotaxis, swim speed, path length, and performance on probe trials. The Greenhouse–Geisser correction was used on all analyses to correct for heterogeneity of variance within groups. For all tests, significance was determined by $p<0.05$.

3. Results

3.1. Developmental measures

There were no significant differences noted in maternal weight gain during gestation [$F(1,5)=0.79$, $p>0.05$] or in litter size [$F(1,5)=0.087$, $p>0.05$] between nicotine treated and control dams. The analysis of birth weight indicated no effect of treatment on nicotine-exposed pups versus controls [$F(1,56)=1.36$, $p>0.05$], and there were no significant differences in weight gain from birth to weaning (P1–P21) between groups [$F(1,54)=.012$, $p>0.05$].

Weight gain from P23–P60 was analyzed by repeated measures ANOVA. There were no significant differences in weight gain between nicotine-exposed males and control males [$F(1,29)=3.26$, $p>0.05$]. However, prenatal nicotine exposure did significantly affect weight gain in females [$F(1,25)=11.60$, $p<0.05$]. As shown in Fig. 1, differences between nicotine-exposed and control females became more pronounced at P45 [control mean=162.9 g; nicotine mean=154.0 g] and gradually increased to a difference of roughly 22 grams at P60 [control mean=216.0 g; nicotine mean=194.3 g].

3.2. Morris water maze

Analysis of behavior in the MWM by nested ANOVA did not show any effects of litter membership on swim speed, path length, latency, or thigmotaxis.

Analysis using a repeated measures nested ANOVA revealed significant differences in swim speeds between control and nicotine-exposed males [$F(1,25)=11.11$, $p<0.05$], with nicotine-exposed males swimming slower than controls. Differences in swim speed among female rats were not significant [$F(1, 21)=0.22$, $p>0.05$]. See Fig. 2 for mean swim speeds across A and B trials. Analysis of swim speed on probe trials was not significant for males [$F(1,25)=0.53$, $p>0.05$] or females [$F(1,21)=2.72$, $p>0.05$].

Analysis of path length revealed significantly longer swim distances in nicotine-exposed females compared to controls [$F(1,21)=4.40$, $p<0.05$]. Mean differences in path length between groups are shown in Fig. 3. There were no significant differences in path length among males [$F(1, 25)=0.44$, $p>0.05$].

Analysis of spatial memory, measured as latency to reach the hidden platform, showed a significant main effect of dose condition across all days of testing in which nicotine-exposed females had the longer latencies [$F(1,21)=4.79$, $p<0.05$] (Fig. 4). The longer latencies in nicotine-exposed females indicated a deficit in reference memory so A trials were analyzed separately. These results show a clear deficit in memory for the location of the hidden platform [$F(1,21)=5.84$, $p<0.05$] (Fig. 5). There were no significant differences on latency among males [$F(1,25)=1.97$, $p>0.05$]. See Fig. 4 for mean latency across A and B trials.

There were no significant differences found between groups in thigmotaxicity during A and B trials for males [$F(1,25)=2.23$, $p>0.05$] or females [$F(1,21)=0.40$, $p>0.05$].

There were no significant differences found between groups on percent time spent in the correct quadrant during probe trials [Males: $F(1,25)=0.65$, $p>0.05$; Females: $F(1,21)=0.18$, $p>0.05$], in the number of platform crossings during probe

Table 1
Probe trial results

Dose condition		Quadrant		Crossings		Latency	
		Mean	SEM	Mean	SEM	Mean	SEM
Control							
Male:	Probe 1	39.1	1.32	3.9	0.46	20.3	2.83
	Probe 2	43.8	2.59	5.2	0.53	15.7	2.70
	Probe 3	51.1	3.40	5.6	0.64	6.9	2.04
	Probe 4	55.2	2.29	6.2	0.46	12.0	2.60
Female:	Probe 1	37.5	3.29	3.8	0.54	21.2	2.88
	Probe 2	47.9	3.02	4.7	0.46	11.4	3.15
	Probe 3	54.5	2.74	5.6	0.47	8.7	1.71
	Probe 4	62.4	3.24	6.4	0.55	10.6	2.63
Nicotine							
Male:	Probe 1	38.3	1.45	3.5	0.27	18.0	2.48
	Probe 2	46.6	2.12	4.0	0.41	14.4	2.15
	Probe 3	52.4	2.26	5.5	0.55	7.0	1.71
	Probe 4	61.7	1.85	5.6	0.42	8.5	1.84
Female:	Probe 1	39.9	3.38	3.8	0.48	19.6	3.09
	Probe 2	46.5	2.88	5.4	0.56	14.3	2.32
	Probe 3	51.1	3.42	5.9	0.57	9.9	2.96
	Probe 4	59.1	3.08	6.9	0.67	11.3	2.86

There were no significant differences between groups on the probe trials. Probe trials were run as the final trial on days 2–5 of testing. "Quadrant" refers to the percentage of time spent searching the correct quadrant during the probe trial. "Crossings" indicates the number of times the rat swam directly over the location of the platform on A and B trials. "Latency" is the time to reach the hidden platform at the end of each probe trial.

trials [Males: $F(1,25)=0.16$, $p>0.05$; Females: $F(1,21)=0.67$, $p>0.05$], or in latency to reach the platform at the conclusion of probe trials [Males: $F(1,25)=1.71$, $p>0.05$; Females: $F(1,21)=0.40$, $p>0.05$]. Means and standard errors for the Atlantis platform trials are indicated in Table 1.

4. Discussion

Our results indicate prenatal nicotine exposure results in long-lasting, sex specific deficits in spatial reference memory as measured by learning in the MWM. Performance of nicotine-exposed female rats on the A and B trials was impaired across five days of testing relative to controls; however, performance on the probe trials was equal between groups, indicating intact working memory. These deficits in cognitive function persist despite a lengthy period of abstinence from nicotine exposure. Additionally, we found a long-term effect of prenatal nicotine exposure on weight gain, which is sex specific and only manifests itself following the hormonal changes associated with puberty.

Previous research in rats has indicated that nicotine exposure towards the end of prenatal development reduces performance on cognitive tasks (Levin et al., 1996). During the late prenatal period and early postnatal period, telencephalic regions important in learning and memory, including the cortex and hippocampus, are differentiating and receptor locations forming, potentially having an influence on future behavior (Levin et al., 1996; Lichtensteiger et al., 1988). Results from this study indicate that chronic nicotine exposure during gestation through the early postnatal period, lead to nicotine-induced deficits in spatial reference memory of female rats, but not in spatial working memory on the MWM task. The increased latencies and path length of nicotine-exposed females indicate a nicotine-induced deficit in acquisition of spatial reference memory. Unlike the A and B trials, performance on the probe trials requires intact spatial working memory to remember the location of the hidden platform between trials rather than across several days of testing. Nicotine-exposed females were not impaired in spatial working memory performance on this task.

It has previously been shown that both acute and chronic nicotine administration have selective effects on improving working memory in adult rats tested in the radial-arm maze but very little affect on reference memory (Levin et al., 1997, 1998). It is known that interactions between the cholinergic and glutamatergic systems are necessary for memory function, and perhaps alterations in nAChR function caused by prenatal exposure to nicotine resulted in downstream effects on glutamatergic neurotransmission, leading to the observed deficits in reference memory but not working memory.

In the current study, nicotine-exposed males had significantly slower swimming speeds in the MWM compared to control males. The alterations in motor behavior seen in nicotine-exposed males seem to indicate disruptions to the mesoaccumbens DA pathway (Oliff and Gallardo, 1999); however, more work is needed to determine the mechanism of this behavioral change.

Activation of the cholinergic system occurs early in prenatal development. Nicotine can interfere with this process leading to structural, chemical, and functional changes in the cholinergic system that are likely to be long-lasting. Previous studies have indicated an increase in nAChRs lasting through the early postnatal period, which return to normal levels in the prenatally exposed rat around P30 (Ernst et al., 2001; Slotkin, 1998). The behavioral differences noted here, suggest that the upregulation of nAChRs during prenatal and early postnatal development may lead to downstream neuronal alterations, which affect specific behaviors including spatial memory. In addition, these findings are suggestive of a sex-selective neurochemical effect of prenatal nicotine exposure.

Chronic exposure to prenatal and early postnatal nicotine at a relatively low dose had no effect on maternal weight gain, litter size, or weight gain in pups from P1–P22. However, weight gain during adolescence in the nicotine-exposed females followed a different trajectory. Nicotine-exposed females differed significantly from control females at such extreme levels, that weight gain from P45–P60 lagged behind controls by as much as 10%. These long-lasting changes, and their timing with respect to sexual maturation, suggest a cascade of effects resulting from developmental nicotine that may interact with later hormonal events of puberty to suppress postpubertal weight gain. While the average weight gain in nicotine-exposed adolescent males was consistently lower than control males, the differences were not significant nor of the magnitude observed in females.

Route of administration is of critical importance in any animal model looking at drug effects. Methodologies for delivering drugs to the neonate rat often involve subcutaneous injections, which are extremely stressful, making it difficult to separate drug effects from the stress of injection. Exposure to nicotine through suckling provides a more natural route of administration. In addition, the gradual decrease in pumping rate of the exhausted Alzet mini-pump minimizes the withdrawal symptoms associated with abrupt dose termination. While the effects of withdrawal on both the dam and pups were not examined in this study, no unusual behaviors were noted when chronic administration ended.

These results support evidence for long-term behavioral and developmental effects induced by prenatal and early postnatal exposure to nicotine that persist into adolescence and adulthood. More research is needed to examine changes in catecholaminergic neurotransmitter systems to determine what changes affecting learning and memory, persist long-term. Taken together, these differences in weight gain and acquisition of spatial learning in the MWM, suggest sex-dependent differences following prenatal nicotine exposure, which should be examined further.

Acknowledgements

Research supported by the Virginia Tobacco Settlement Foundation. The authors wish to thank Dr. Craig G. McDonald for helpful comments during preparation of the manuscript.

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