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Paternal nicotine exposure in rats produces long-lasting neurobehavioral effects in the offspring

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ABSTRACT

Studies of intergenerational effects of parental chemical exposure have principally focused on maternal exposure, particularly for studies of adverse neurobehavioral consequences on the offspring. Maternal nicotine exposure has long been known to cause adverse neurobehavioral effects on the offspring. However, paternal toxicant exposure has also been found to cause neurobehavioral toxicity in their offspring. Recent work suggests that paternal nicotine exposure can have epigenetic effects, although it remains unclear whether such changes lead to neurobehavioral effects. In the current study, we investigated the effects of paternal nicotine exposure on neurobehavioral development of their offspring. Male Sprague-Dawley rats were exposed to 0 or 2 mg/kg/day nicotine (sc) for 56 consecutive days with two consecutive 2ML4 osmotic minipumps. Following treatment, these males were mated with drug-naïve female rats. Offspring of both sexes were tested in a behavioral battery to assess locomotion, emotional function and cognition. Paternal nicotine exposure did not impact offspring viability, health or growth. However, behavioral function of the offspring was significantly altered by paternal nicotine exposure. Male offspring with paternal nicotine exposure exhibited locomotor hyperactivity in the Figure-8 apparatus when tested during adolescence. When retested in adulthood and regardless of sex, offspring of the nicotine exposed father showed significantly reduced habituation of locomotor activity over the course of the session. Compared to controls, female offspring of nicotine-exposed fathers showed significantly reduced response latency in the radial arm maze test. In addition to locomotor hyperactivity, the offspring of nicotine-exposed fathers also showed significantly diminished habituation in the novel object recognition test. These results indicate that chronic paternal nicotine exposure can impact the behavior of offspring, producing locomotor hyperactivity and impaired habituation.

1. Introduction

Parental tobacco smoking is a major risk factor for offspring health and is associated with a variety of neurobehavioral effects, including increased risk for psychiatric disorders and alterations in cognitive and affective functions (Vassoler et al., 2014; He et al., 2017; Meier et al., 2017; Sciberras et al., 2017). Studies investigating how this risk is conferred to offspring have largely focused on how tobacco smoke constituents impact the developing brain, linking maternal smoking during pregnancy to adverse outcomes later in life. One of the primary neurotoxins of concern in tobacco smoke is nicotine, which is found in both combustible cigarettes and alternative products such as smokeless tobacco and e-cigarettes. Prenatal exposure to nicotine produces neurobehavioral effects in animal models which mirror psychiatric effects seen in children of tobacco smokers (Hall et al., 2016; Schneider, 2017). Maternal nicotine exposure during pregnancy may then account for some of the risk presented by parental smoking, although these are not the only mechanisms likely to be involved. Advances in epigenetic analyses of toxicity have identified additional mechanisms that may be capable of altering neural development and impairing behavioral health.

Of particular interest, epigenetic toxicity studies have observed that exposure to tobacco products can alter the methylation patterns of DNA. Active adult smokers exhibit changes in methylation at hundreds of CpG sites (Zeilinger et al., 2013) and certain effects may persist for decades (Ambatipudi et al., 2016). Sperm are also vulnerable to these effects (Jenkins et al., 2017) raising concern that smoking-induced methylation changes in males may be passed on to their offspring

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(Soubry et al., 2014). Although the majority of genomic DNA methylation in sperm is removed after fertilization, some methylation is retained (Tang et al., 2015). Any remaining methylation could be passed on to the offspring and alter gene regulation and expression. Recently, Jenkins et al. (Jenkins et al., 2017) reported widespread effects of smoking on methylation in the sperm of male participants. More specifically, smoking was shown to alter methylation at diverse CpGs or within specific regions of the genome, as well as generally increasing the variability in genome-wide methylation patterns (Jenkins et al., 2017).

Although it has been observed that sperm may be epigenetically altered by nicotine exposure, the impacts of these changes on brain development and behavioral health remain poorly understood. Previous studies have shown relatively specific and subtle effects of paternal exposure to drugs of abuse such as nicotine, alcohol, psychostimulants and opiates (Goldberg and Gould, 2018). In particular, these exposures have the potential to alter the response to these or other drugs of abuse in the offspring, either enhancing or attenuating those responses in subsequent generations (Vyssotski, 2011; Finegersh and Homanics, 2014; Fischer et al., 2017; Rompala et al., 2017). Changes in baseline behavior, as would be relevant to developmental disorders, have also been reported, although they tend to be specific and impact certain tests while sparing others (Yohn et al., 2015a, 2015b; Goldberg and Gould, 2018). A handful of these studies have examined paternal nicotine exposure in mice (Dai et al., 2017; Vallaster et al., 2017; McCarthy et al., 2018; Yohn et al., 2018), suggesting that certain behaviors, such as locomotor activity, may be sensitive to paternal nicotine effects. Overall though, data on paternal nicotine-induced changes in baseline behaviors remain limited.

Based on previous studies, it was hypothesized that chronic paternal exposure to nicotine during spermatogenesis would result in heritable behavioral effects in the offspring and that these effects will be specific rather than general, impacting certain functional areas while sparing others. The present study was conducted using a multifaceted behavioral battery to assess the profile and specificity of paternal nicotine effects in a rodent model. Male breeder rats were passively exposed to nicotine (2 mg/kg/day) or vehicle through osmotic minipumps over a 56-day period prior to mating. This period ensured that exposure would cover the length of a full spermatogenic cycle in rats. Male and female offspring were reared and tested in a behavioral battery which spanned from juvenile to young adult development. Behavioral assays were selected to measure a range of locomotor, affective and cognitive functions.

2. Methods

2.1. Design

The subjects in this study were young adult male Sprague-Dawley rats (Charles Rivers Labs, Raleigh, NC, USA) (200-250gr). Chronic exposure to nicotine detartrate was delivered via osmotic minipump (Alzet model 2ML4, Durect Inc., Cupertino, CA, USA) at 2 mg/kg/day (dose calculated as of the nicotine base weight). These minipumps were rated to deliver consistent exposure for 28 days, so two consecutive minipumps were implanted to cover a total duration of 56 days. These minipumps were placed on opposite flanks of the body. For controls, the same surgery and pumps were used, but the pumps contained only the saline vehicle. During the surgery, animals were anaesthetized using ketamine (60 mg/kg) and dexdormitor (15 mg/kg). At the end of 56 days, the remaining pump was removed and allowed a recovery period of 72 h prior to mating to avoid post-operative stress and nicotine withdrawal effects during the mating period. These male rats were then individually housed with drug naïve young adult female Sprague-Dawley rats from the same vendor for mating. A total of eighteen breeding pairs, split among the two treatment groups, were used. Two dams matched with control studs failed to become pregnant. These studies were conducted under a protocol approved by the Institutional Animal Care and Use Committee of Duke University and meet the requirements of state, federal and international regulatory bodies. All animals had ad libitum access to food and water, except in select food-motivated testing (see below) and were maintained on a 12/12 reversed day-night cycle.

2.2. Clinical effects

Body weights were recorded weekly for the females after mating and throughout gestation. After parturition, the offspring were also weighed weekly. After weaning, one male and one female from each litter were kept for behavioral assessment.

2.3. Housing and behavioral testing

Animals used in the behavioral study were weaned at 21 days old and 1 male and female rat was randomly selected for inclusion in behavioral testing. These offspring were housed with 1–2 same-sex rats of the same treatment for the duration of testing. These animals were allowed to acclimate to these conditions for one week prior to the start of the behavioral battery, which consisted of assays for locomotor activity, emotional function and cognition (see Table 1). All subjects completed all tests in the battery. Daily testing was performed between 9 and 17 h, which was during the dark phase of the reversed day-night cycle. All tests were performed under low ambient light conditions. Ad libitum food access was maintained except during novelty suppressed feeding, radial arm maze and the operant signal detection testing sequences. For novelty suppressed feeding, food was removed 24 h before testing to stimulate feeding. The rats were returned to ad libitum food access after the session. For radial arm maze and operant signal detection testing, rats were maintained on a restricted diet to maintain them at ~85% of free feeding body weight.

2.4. Week 4: elevated plus maze

At 4 weeks of age, male and female offspring were tested in the elevated plus maze (Med Associates, St Albans, VT, USA) to assess their anxiety-like behavior vs. risk-taking behavior (as in Hall, 2016). The maze (142-cm × 104-cm × 76-cm high) was made of black Plexiglas and consisted of two arms with 15-cm high enclosed walls and two open arms with 2-cm railings. Each rat was allowed to explore the elevated plus maze for a single five-min session. Rats show a species-typical preference for closed arms over open arms. Anxiety-like or risk-taking behavior was assessed as the percentage of time the rat spent in the open vs. enclosed arms of the maze. Additionally, the number of crossings through the center junction was counted as a measure of activity.

2.5. Week 5: Figure-8 apparatus test of locomotor activity

At 5 weeks, offspring were tested for locomotor activity and

Table 1
Sequence and testing ages for behavioral testing.

AGE	4wk	5wk	6wk	7wk	8–11 wk	11 wk	12 ± wk	Adult
Test	Elevated plus maze	Figure 8 maze	Novelty suppressed feeding	Novel object recognition	Radial arm maze	Figure 8 maze	Signal detection test	Figure 8 maze

habituation in the Figure-8 maze (as in Hall, 2016). The Figure-8 apparatus consisted of a continuous alley measuring 10-cm × 10-cm in the shape of a Figure-8 with two side alleys. The entire maze was enclosed and measured 70-cm × 42-cm. The rats were allowed to explore the apparatus for a single, one-hour test session, during which their movements were detected by eight photo-beams located at approximately equal points throughout the maze. Photo-beam breaks were analyzed across time in twelve 5-min blocks. The mean number of photo-beam breaks per 5-min block was used as an index of locomotor activity. The linear trend of decreasing beam breaks across the 5-min time blocks was used as an index of the habituation of activity. In order to track locomotor activity effects across development, subjects were also tested in the Figure-8 maze as young adults (week 11) and full adults (following completion of attention task).

2.6. Week 6: novelty suppressed feeding

At 6 weeks, offspring were tested for fear responsivity using the novel environment suppressed feeding test (as in Hall et al., 2016). Following 24-h of food restriction, rats were individually placed in a plastic cage matching the dimensions of their home cage. This cage did not have a lid or bedding and was located in the middle of a brightly lit testing room. Twelve standard rat-chow pellets were weighed before testing and were laid on the floor in 4 rows of 3 pellets each. The rats were allowed to remain in the cage and consume the food for 10 min. Under these conditions, rats typically show low levels of food consumption and long latencies to consume food relative to familiar or home cage environments, indicating a stress or fear-like response to these cues. Eating was defined as the act of chewing the food. The food pellets were weighed before and after the session to determine total food consumption. Dependent measures included the total food consumed, the latency to begin eating, the number of eating bouts and the duration of eating.

2.7. Week 7: novel object recognition

At 7 weeks, offspring were tested for attention and recognition memory using the novel object recognition test (as in Hall et al., 2016). Recognition of a novel vs. familiar object was used to test attention and memory in a low-motivation state. Rats were habituated in an opaque plastic enclosure (70-cm × 41-cm × 33-cm) over two 10-min sessions in two days. On day 3, animals were placed in the enclosure and allowed to explore two identical objects (A/A) made of plastic, glass, or ceramic material for 10-min. The objects were randomized for each animal. After this A/A session, rats were returned to their home cage for 1-hr before the second (A/B) session. In the A/B session, the enclosure contained two different objects, one object from the A/A session and another, visually distinct "novel" object (A/B session) of the same material. Any scent cues from previous interactions were removed by wiping down each of the objects with a vinegar solution prior to the session. The test session lasted for ten min. Interaction with the novel and familiar object in the A/B session was measured as the total number of second spent interacting with each, then as a preference between the two objects. These data were analyzed in the first and second halves of this testing window to detect preference between the objects when the novel object is highly unfamiliar (first 5 min) and after the rat has gained more experience with the novel object (second 5 min). Analysis considered the preference in the first and second halves of the sessions.

2.8. Week 8–11: radial-arm maze

Beginning at 8 weeks of age, offspring were tested in the radial arm maze, a test of spatial learning, short term and long term memory (as in Hall et al., 2016). The maze was made of black painted wood and consisted of 16 arms (10 cm × 60 cm) radiating from a central hub (50 cm diameter). During testing, rats were individually placed into the maze and allowed to explore the arms and retrieve half-pieces of sugar-coated cereal (Froot Loops®; Kellogg's Inc., Battle Creek, MI, USA) from food cups located at the distal end of the arms (2 cm from end). Navigation was aided by visual cues (cardboard shapes) placed on the walls of the testing room. Prior to training in the maze, the rats underwent two 10-min habituation sessions where they were blocked in the central hub and allowed to consume pieces of the cereal rewards. Following this, subjects completed 18 daily test sessions in the maze, where the maze had 12 baited arms containing cereal pieces and 4 arms containing no food (Hall et al., 2016). The locations of the baited arms were randomized across subjects, but remained constant for each subject throughout training. Each rat was placed on the central hub in a plastic cylinder for 10 s prior to the start of the trial, at which point the cylinder was removed. The trial ended after 10 min or all twelve baited arms were visited, whichever occurred first. Arm entries were assessed for working and reference memory errors. Any entry into an arm which was previously entered on the same session was scores as a working memory error. Any entry into am arm which was left unbaited throughout training was scored as a references memory error. Latency, or response rate, was calculated as the total session time divided by the number of arm entries.

2.9. Weeks 12–40: operant visual signal detection task for attention

The operant visual signal detection test was completed as described by Hall et al. (Hall et al., 2016). Testing was conducted in an operant chamber containing two retractable levers and a cue light. Through the training sequence, each rat was trained to press one of two retractable levers according to the presentation of a cue-light illuminated for 500 ms, or its absence. If the cue-light was illuminated prior to the presentation of the two levers ("signal" trial), lever presses on the corresponding "signal" lever resulted in the delivery of a 20 mg sugar pellet. If the levers were presented without a cue-light presentation ("blank" trial), lever presses on the opposite lever resulted in the delivery of a 20 mg sugar pellet. The spatial arrangement of the "signal" and "blank" levers was counterbalanced across the rats. If no response was made within 5 s of lever presentation, both levers retracted and a response "failure" was recorded. Each session in the testing phase included 240 total trials, with equal numbers of "signal" and "blank" trials. Correct responses were scored as "hits" on signal trials and "correct rejections" on blank trials. Dependent measures included percent correct hit and percent correct rejection per session. Data analysis addressed hits, correct rejections and failures.

2.10. Data analysis

For each behavioral test, data was analyzed via mixed-factors analysis of variance (ANOVA). As one male and one female were selected from each litter (i.e. each exposure), litter was the primary unit of variance. Sex was the within-litter variable of interest. Paternal treatment was the between-litter variable of interest. Repeated measures included age of testing (Figure 8 maze was tested at three ages), session, or time block within session, as pertinent to each test. Follow-up analyses were then performed based on the main effects or interactions detected by the omnibus test, consisting of a simpler ANOVA and/or pairwise comparisons, labeled as simple main effects. In the omnibus test, follow-up tests were performed on interactions at $p < 0.1$ (Snedecor and Cochran, 1967), although a cut-off of $p < 0.05$ (two-tailed) was used as the threshold for main effects and final statistical significance of post hoc values. Significant results are reported in the result section (see below). A full reporting of analyses are included in Table 1.

3. Results

3.1. Clinical signs

No effects of paternal nicotine exposure were seen in measures of clinical health. Mixed factors ANOVAs for maternal (paternal nicotine treatment × time) and offspring (sex × paternal nicotine treatment × time) body weight showed that maternal and offspring growth were not significantly affected by paternal nicotine exposure. Univariate analyses of litter size and sex distribution similarly showed no alterations due to paternal nicotine treatment.

3.2. Elevated plus maze

Univariate analyses showed no significant effects of paternal nicotine exposure on the percent of the time spent in the open arm time or the number of center crossings in the elevated plus maze test.

3.3. Locomotor activity in the Figure-8 apparatus

Mixed factors ANOVA revealed a significant age × paternal nicotine × sex interaction of locomotor activity in the Figure-8 maze ($F(2,24) = 4.13$, $p < 0.05$), which was followed by mixed factors analyses at each of the ages (paternal nicotine treatment × sex × time block). Locomotor activity in adolescent offspring showed a significant nicotine × sex interaction ($F(1,13) = 6.26$, $p < 0.05$). This prompted follow-up tests of the simple main effects of paternal nicotine separately in each sex (Fig. 1). Paternal nicotine exposure caused a significant ($p < 0.025$) degree of locomotor hyperactivity in juvenile male offspring (41.1 ± 1.7) vs. male controls (34.5 ± 1.7). In contrast, no significant effects were seen in females (Control = 38.1 ± 1.8, Nicotine = 37.1 ± 2.4). The rats were re-tested for locomotor activity in the Figure-8 apparatus two more times: during young adulthood and as adults. In the young adult test, mixed factors analysis revealed a was a significant paternal nicotine treatment × time block within session interaction ($F(11,143) = 1.92$, $p < 0.05$). Tests of the simple main effects showed a pattern of slower habituation of locomotor behavior by the offspring of the nicotine-treated males (Fig. 2). Simple main effects were further performed in each time block, revealing that paternal nicotine exposure caused significantly increased activity during time blocks 4 ($p < 0.005$), 6 ($p < 0.05$) and 7 ($p < 0.01$), with a nearly significant increase during time block 5 ($p < 0.06$). In the third locomotor activity test during full adulthood, there was a significant three-way (sex × paternal nicotine × time block) interaction ($F(11,132) = 1.92$, $p < 0.05$), but the simple main effects tests of paternal nicotine exposure did not detect any significant paternal nicotine effects for males or females at any of the time blocks within the session (Fig. 3).

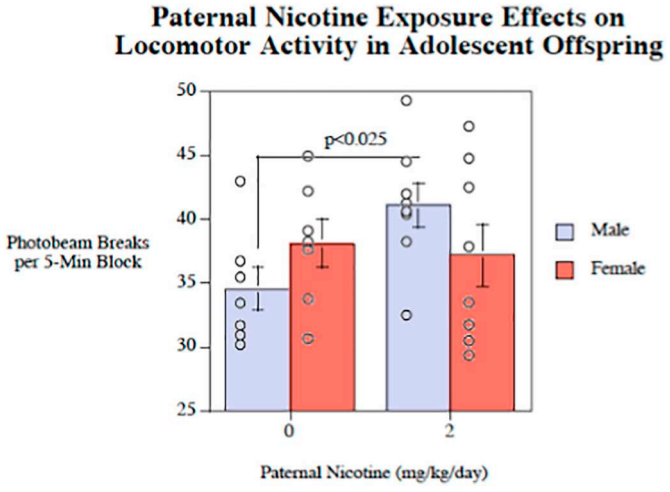

Fig. 1. Adolescent locomotor activity in the Figure-8 maze (mean ± sem). There was a significant paternal nicotine × offspring sex interaction. Paternal nicotine exposure caused significant locomotor hyperactivity in the male offspring ($p < 0.025$); female locomotor activity was not found to be affected by paternal nicotine exposure. Circles indicate individual subjects.

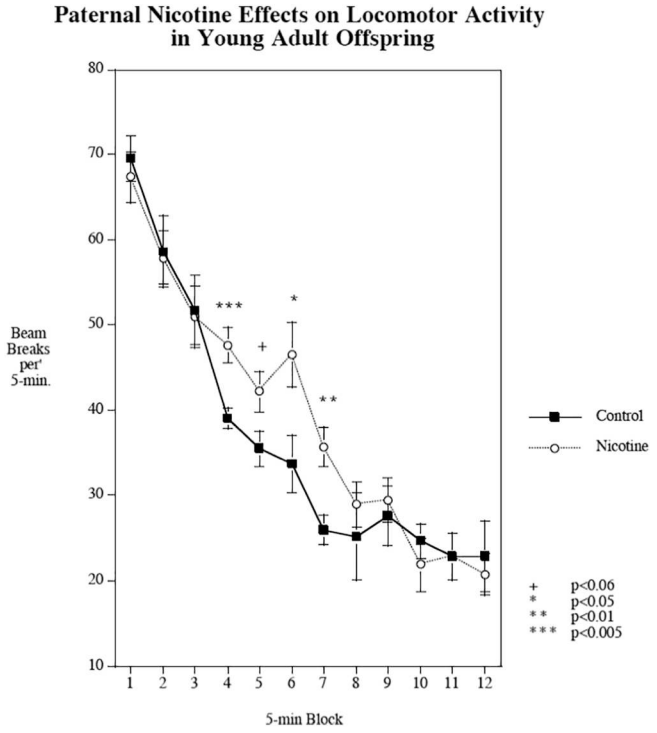

Fig. 2. Young adult locomotor activity in the Figure-8 maze (mean ± sem). There was a significant interaction of paternal nicotine treatment × 5-min time block within the 1-h session. A slower habituation of locomotor activity was noted in offspring of nicotine exposed males, as evidenced by significantly elevated activity in time blocks 4, 6 and 7 ($p < 0.05$), and a marginally significant elevation in block 5 ($p < 0.06$).

3.4. Novelty suppressed feeding

Univariate ANOVAs revealed no significant paternal nicotine effect feeding in the novelty suppressed feeding test. This included latency to begin feeding, amount of food eaten, number of feeding bouts and the total duration of feeding (Table 2).

3.5. Novel object recognition

Novel object recognition was analyzed through mixed factors ANOVA. Overall, rats showed greater investigation of the novel object than the familiar object ($F(1,13) = 8.71$, $p < 0.025$), indicating that the rats generally discriminated between the objects based on familiarity (Fig. 4). However, a significant three-way (paternal nicotine × familiar/novel objecttime block) interaction was also observed ($F(1,13) = 6.89$, $p < 0.025$). To probe this interaction, analyses of the simple main effects during each 5 min time block were performed. Controls showed a typical pattern for this task, as they significantly preferred the novel object during the first 5 min of the test ($p < 0.01$), then showed a significant ($p < 0.025$) drop-off in investigation of the novel object during minutes 6–10 of the test as the previously novel object became familiar. In contrast, the offspring of the nicotine-treated fathers did not show a robust preference for the novel object during the first half of the test ($p < 0.08$) but did have a significant ($p < 0.01$) preference for the novel object during the second half of the test.

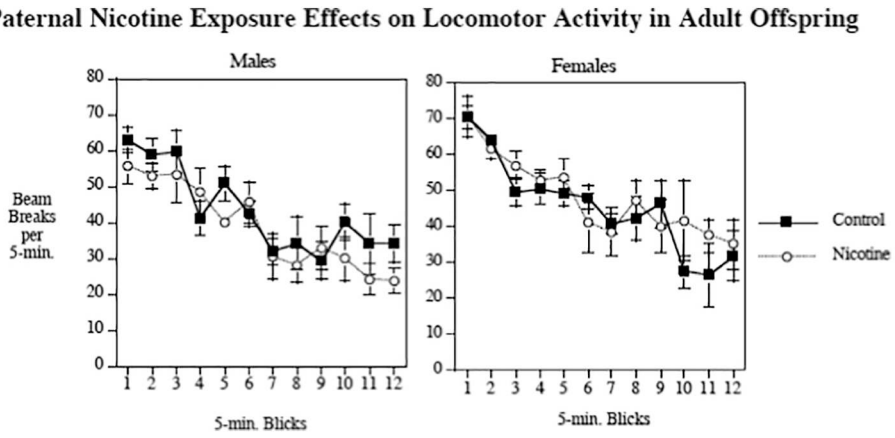

Fig. 3. Adult locomotor activity in the Figure-8 maze (mean ± sem). There was a significant three-way interaction of paternal nicotine × sex of the offspring × 5-min time block within the 1-h session. However, none of the simple main effects of paternal nicotine exposure were significant.

3.6. Radial-arm maze

Mixed factors analysis of variance detected no significant effects of paternal nicotine treatment on error rates in the radial-arm maze. Two other main effects were detected which demonstrate that the subjects' performance was typical for the task. There was a significant main effect of session block ($F(3,39) = 3.52, p < 0.025$) with improvement as training progressed. There was also a significant effect of error type ($F(1,12) = 321.78, p < 0.0005$) with fewer reference than working memory error types committed.

An additional factor of interest was latency, or the amount of time spent per arm entry. Mixed factors ANOVA demonstrated a significant interaction of paternal nicotine × sex ($F(1,13) = 7.02, p < 0.025$). Follow-up tests of the simple main effects of paternal nicotine in each sex showed that female offspring of nicotine-treated males were significantly ($p < 0.001$) faster than control females while there was no significant paternal nicotine effect in male offspring (Fig. 5). There was also a significant ($F(3,39) = 75.88, p < 0.0005$) main effect of session block, reflecting a steady decrease in latency over the course of training. This is typical with radial-arm maze training.

3.7. Signal detection attention task

Mixed factors analysis of variance detected no significant effects of paternal nicotine treatment on performance in the signal detection attention task, either as a main effect or interaction with the other factors of sex, trial type or session. Two other main effects were detected which demonstrate that the subjects' performance was typical for the task. There was a significant effect of session ($F(5,60) = 56.60, p < 0.0005$) with choice accuracy continually improving over the six sessions of training. There was also a significant effect of trial type ($F(1,12) = 35.33, p < 0.0005$) with the typical better performance on correct rejection trials than hit trials.

4. Discussion

The current study showed that chronic paternal nicotine exposure prior to mating, which modeled nicotine exposure in moderate tobacco use, caused significant, long-lasting behavioral effects in the offspring. In particular, locomotor hyperactivity was apparent following paternal nicotine exposures. Additionally, paternal nicotine exposure led to alterations in the investigation of novel objects and environments over time and response speed in the radial arm maze. Paternal treatment effects appeared to be relatively specific inasmuch as behavioral measures of activity and habituation were impacted, while tests of emotional function and cognition were spared. These patterns of hyperactivity and impaired adaptability are concerning, as similar problems are often seen in children with attention deficit hyperactivity disorder, a disorder which is associated with parental smoking (Biederman et al., 2017; Huang et al., 2017)

Of the measures assessed in this study, locomotor activity appeared to be particularly sensitive to paternal nicotine exposure effects. Paternal nicotine exposure generally produced hyperactivity in offspring, although this effect varied considerably across development. Among adolescent offspring, there was a sexually dimorphic effect, whereby males showed hyperactivity while females were unaffected. This dimorphism did not persist into adulthood. Young adult offspring of nicotine-exposed males showed a more selective increase in activity during the middle of the session, with no change in maximum or minimum activity levels early and late in the session. By full adulthood, no alterations were apparent. These data show that paternal nicotine exposure may lead to hyperactivity in offspring but that these effects may not appear equally in male and female subjects, and may attenuate over the course of development. It is notable that adolescent rats showed a sex difference in vulnerability to paternal nicotine-induced hyperactivity, although there is limited evidence to suggest why males may be more susceptible to paternal exposure effects than females. Future research should evaluate the impacts of paternal nicotine on sexually-dimorphic factors which could preferentially affect males, including genetic or neuroendocrine factors. Additionally, these sex differences should be investigated in the context of adolescent development. The attenuation and eventual elimination of hyperactivity suggests that developmentally typical processes may alleviate these alterations. So, the male-specific hyperactivity in adolescence could indicate a developmental delay in those processes among males, rather than a uniquely male risk factor. Such a developmental delay could represent either an additional epigenetic mechanism or a typical sex difference. A more detailed temporal analysis of these transitions may better demonstrate when and under what circumstances these sex differences are expressed and what range of development will be most impacted by hyperactivity effects.

In addition to hyperactivity, paternal nicotine exposure led to impairments in habituation, a basic learning process which reduces responding across repeated or extended exposure to a stimulus. In the Figure-8 maze, activity levels are reduced across the session as rats acclimate to the new environment and explore less. Similarly, rats in the novel object recognition task show a strong preference for a novel object over a familiar one, with that preference reducing over time as the novel object is repeatedly investigated. Offspring of nicotine-exposed males showed altered patterns of habituation in both of these tasks. Young adult offspring of nicotine-exposed males showed slower locomotor habituation, indicating that these animals required greater time or exploration to achieve the same reduction in activity shown by controls. Similarly, adolescent offspring of nicotine-exposed males

Table 2
Statistical analyses of behavioral tests of the offspring.

Elevated plus maze			
Percent open arm time	*p*-Value	Mean percent	
		Control	NIC
NIC	0.55	43.9	39.6
		Male	Female
Sex	0.22	37.1	46.1
NIC × Sex	0.70	Male	Female
Control		38.0	49.9
NIC		36.4	42.8
Center Crosses	p-Value	Mean number	
		Control	NIC
NIC	0.99	4.4	4.4
		Male	Female
Sex	0.77	4.3	4.6
NIC × Sex	0.22	Male	Female
Control		4.9	4.0
NIC		3.8	5.1

Figure-8 activity				
Overall	p-Value	Mean beam breaks/5-min block		
		Control	NIC	
NIC	0.50	39.0	40.7	
		Adolescent	Juv. adult	Adult
Age	0.002	38.0	37.7	43.9
		Male	Female	
Sex	0.13	38.6	41.1	
NIC × Sex	0.84	Male	Female	
Control		37.6	40.5	
NIC		39.5	41.8	
5-min Block	0.0001			
NIC × Age × Block	0.03			
Adolescent	p-Value	Mean beam breaks/5-min block		
		Control	NIC	
NIC	0.25	36.3	39.1	
		Male	Female	
Sex	0.91	38.0	38.0	
NIC × Sex	0.03	Male	Female	
Control		34.5	38.1	
NIC		41.1	37.1	
5-min block	0.0001			
		Mean beam breaks/5-min block		
nic × trial block	0.75	Control	NIC	
1		58.3	57.1	
2		47.5	57.0	
3		44.4	48.6	
4		42.1	43.0	
5		39.6	40.6	
6		36.0	37.8	
7		34.9	33.9	
8		28.9	35.5	
9		29.2	31.1	
10		27.1	29.1	
11		26.8	30.4	
12		20.8	25.12	
Juvenile adult	p-Value	Mean beam breaks/5-min block		
		Control	NIC	
NIC	0.22	36.5	39.4	
		Male	Female	
Sex	0.16	36.4	39.6	
NIC × Sex	0.75	Male	Female	
Control		35.2	37.7	
NIC		37.5	41.24	
5-min block	0.0001			
		Mean beam breaks/5-min block		
NIC × Trial block	0.75	Control	NIC	
1		69.5	67.4	
2		58.6	57.8	

(*continued on next page*)

Table 2 (*continued*)

Figure-8 activity			
Overall	p-Value	Mean beam breaks/5-min block	
		Control	NIC
3		51.8	50.9
4		39.0	47.6
5		35.5	42.2
6		33.6	46.6
7		26.0	35.8
8		25.2	28.9
9		27.6	29.4
10		24.6	21.9
11		22.9	22.9
12		22.9	20.8
Adult	p-Value	Mean beam breaks/5-min block	
		Control	NIC
NIC	0.81	45.4	42.8
		Male	Female
Sex	0.06	42.3	45.6
NIC × Sex	0.27	Male	Female
Control		43.2	47.5
NIC		41.5	44.0
5-min Block	0.0001		
		Mean beam breaks/5-min block	
NIC × Trial Block	0.98	Control	NIC
1		66.5	63.1
2		61.4	57.3
3		54.5	55.1
4		45.8	50.6
5		50.0	46.9
6		45.3	43.3
7		36.4	34.5
8		38.2	37.7
9		37.8	36.4
10		33.7	35.7
11		30.2	30.7
12		32.9	29.4

Novelty suppressed feeding	p-Value	Mean seconds eating	
		Control	NIC
NIC	0.39	161.3	185.1
		Male	Female
Sex	0.001	224.1	123.9
NIC × Sex	0.56	Male	Female
Control		203.4	119.1
NIC		242.1	128.0

Novel object recognition	p-Value	Mean seconds investigating	
		Control	NIC
NIC	0.53	47.4	43.4
		Male	Female
Sex	0.07	39.8	50.7
NIC × Sex	0.27	Male	Female
Control		42.3	49.5
NIC		35.0	51.8
Novel vs. Familiar	0.01	Novel	Familiar
		52.8	37.7
5-Min Time Block	0.21	Min 1–5	Min 6–10
		47.8	42.8
NIC × Time × Novel/Familiar	0.02	Min 1–5	Min 6–10
Control - Familiar		40.1	42.8
Control - Novel		62.5	44.2
Nicotine - Familiar		38.4	30.5
Nicotine - Novel		50.9	53.7

Radial-arm maze	p-Value	Mean errors

(*continued on next page*)

Table 2 (*continued*)

Mean errors		Control		NIC	
NIC	0.26	9.2		8.7	
		Male		Female	
Sex	0.17	8.6		9.6	
NIC × Sex	0.91	Male		Female	
Control		8.51		8.76	
NIC		8.32		8.35	
Error type	0.0001	Working		Reference	
		10.9		7.0	
Session block	0.02	1–3	4–6	7–9	10–12
		9.9	8.9	9.2	7.7
Mean response latency	p-Value	Mean response latency			
		Control		NIC	
NIC	0.13	14.3		12.8	
		Male		Female	
Sex	0.52	13.4		13.7	
NIC × Sex	0.02	Male		Female	
Control		13.3		15.4	
NIC		13.5		12.2	
Session block	0.0001	1–3	4–6	7–9	10–12
		19.1	13.3	11.3	10.5

Signal detection attention test	p-Value	Mean percent correct	
		Control	NIC
NIC	0.79	84.8	85.2
		Male	Female
Sex	0.28	85.9	84.1
NIC × Sex	0.60	Male	Female
Control		86.4	83.3
NIC		85.5	84.9
		Hit	Correct rejection
Trial type	< 0.0001	81.1	88.9
NIC × Trial type	0.12	Control	NIC
Hit		81.1	81.0
Correct rejection		88.5	89.3

failed to show the characteristic reduction in preference for a novel object across two 5-min time blocks. Rather, these subjects showed a preference for the novel object which failed to reach significance in the first time block, but was significant in the second time block. This indicates that these subjects recognized the familiar object and preferred a novel one, but that a 10-min session was not sufficient to observe habituation to the novel object in these animals.

The final behavioral alteration observed in this study was in the radial arm maze. Specifically, female offspring of nicotine-exposed males moved from arm to arm more quickly than control females did. This effect was not detrimental to task performance overall, as the error rate was unaffected by paternal treatment, although it may indicate a subtle behavioral effect not related to learning and memory. For instance, faster food retrieval may simply reflect a hyperactivity effect, as indicated by other tasks, or perhaps greater motivation for the food reinforcers. Male offspring of nicotine-treated fathers did not differ from controls on this outcome.

Taken together, the present data suggest that paternal nicotine exposure can have multigenerational effects on neurobehavioral function. Although the literature on paternal exposure effects still has substantial gaps, our data appear to fall in line with existing behavioral studies following paternal drug exposure. Current evidence generally supports the conclusion that commonly abused substances such as ethanol, opiates and psychostimulants can have behavioral effects on the next generation (Yohn et al., 2015a, 2015b; Goldberg and Gould, 2018), although these effects can vary based on species and strain of animal

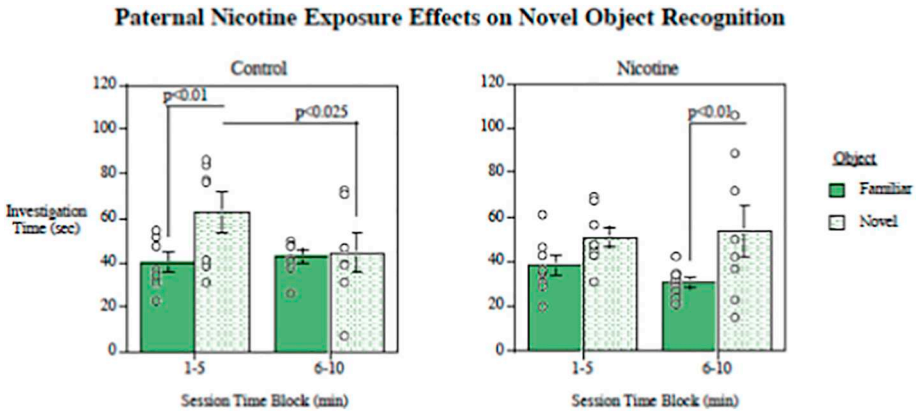

Fig. 4. Novel Object Recognition, time in seconds spent investigating the familiar and novel objects during the first and second five-min time blocks of the session (mean ± sem). There was a significant interaction of paternal nicotine × familiar vs. unfamiliar object × five min time block ($p < 0.025$). Controls showed significant novel object preference ($p < 0.01$) and habituation ($p < 0.025$), while offspring of nicotine treated sires only showed preference in the second time block ($p < 0.01$) Circles indicate individual subjects.

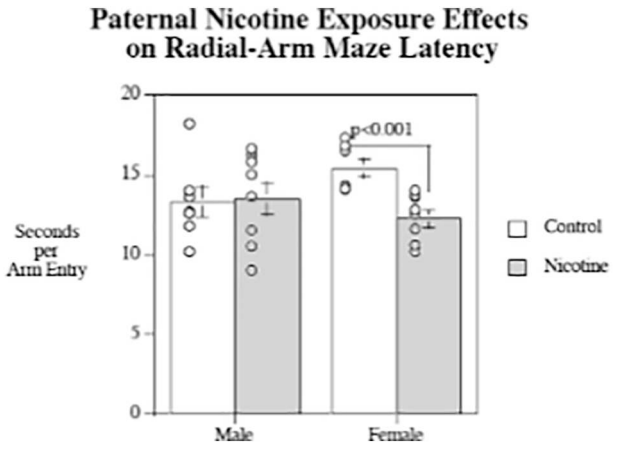

Fig. 5. Radial-arm maze response latency (seconds per arm entry) showed a significant ($p < 0.025$) paternal nicotine × sex interaction with female offspring of nicotine-treated fathers having significantly ($p < 0.001$) longer latencies than control females. No significant paternal nicotine effects were seen in male offspring with this measure (mean ± sem). Circles indicate individual subjects.

used, age tested, sex of the offspring, and dose of exposure. More work is needed to evaluate the potential effects of nicotine. To date, there are no known published studies examining the offspring of nicotine-exposed male rats, although there are a few with mice. Dai et al. (2017) reported that paternal nicotine exposure (0.05 mg/100 g nicotine, i.p., 4× daily for 5 weeks) produced highly selective effects on behavior, leading to locomotor hyperactivity and decreased immobile time in the forced swim test, with all other social, affective and cognitive tests unaffected. These effects were similar to those produced by paternal exposure to inhaled tobacco smoke, though tobacco smoke had an additional effect on social motivation. McCarthy et al. (2018) similarly reported that paternal nicotine exposure (200 µg/mL in water for 12 weeks) led to locomotor hyperactivity and significant deficits in reversal learning, a form of cognitive adaptability, in both male and female offspring. Vallaster et al. (2017) provided voluntary nicotine exposure (200 µg/mL in water for 5 weeks) to males prior to mating. This exposure had some limited effects on nicotine sensitivity in the offspring, but no significant effects on behavior. The present data are generally consistent with these studies, in that certain specific functions, including locomotor activity and adaptability, are impacted by paternal nicotine exposure, while the bulk of behavioral functions are spared. Insensitive outcomes included anxiety and fear-like behaviors, motivated learning, memory and sustained attention.

Additional analyses will be needed to determine the mechanisms by which paternal nicotine exposure causes behavioral alterations in their offspring. Sperm samples were collected from the paternal males (F0) in this study for epigenetic analysis, as were sperm and/or tissue samples from the F1 and F2 generations. Critical epigenetic effects and their respective heritability will be assessed using those samples and published separately. Nicotine-induced alterations in sperm DNA methylation are likely candidate mechanisms for paternally-heritable behavioral dysfunction, as sperm DNA methylation is independently associated with many features of offspring health (Soubry et al., 2014). For example, naturally occurring changes in sperm DNA methylation due to paternal age are associated with elevated rates of offspring behavioral disorders such as schizophrenia and autism spectrum disorders (Milekic et al., 2015). Although more work is needed, early indications suggest that paternal nicotine exposures which alter offspring behavior are similarly associated with changes in sperm DNA methylation (Dai et al., 2017). It is hypothesized that the present findings are associated with altered methylation at sites which modulate risk for neurodevelopmental problems, although other transferrable germline modifications may also need to be investigated in the future (Soubry et al., 2014; Vassoler et al., 2014), such as histone modifications or non-coding RNAs. Further studies should also be undertaken to clarify the importance of nicotine in the context of tobacco smoke, a complex mixture containing thousands of chemicals, many of which are known to be toxic. Comparisons of paternal nicotine with paternal exposure to tobacco smoke (Dai et al., 2017), tobacco smoke extract (Hall et al., 2016), or other individual tobacco smoke constituents may improve our understanding of how shifting trends towards smokeless or electronic tobacco products among men (Anic et al., 2018) may impact behavioral health in future generations.

Abbreviations

CpG	cytosine (C) and guanine (G) with a phosphodiester bond (p)
DNA	Deoxyribonucleic acid
H	hour
I.P.	intraperitoneal
Min	minute
S	second
SEM	standard error or the mean
SC	Subcutaneous

Transparency document

The Transparency document associated with this article can be found, in online version.

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Author contributions

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6) Susan K. Murphy - Conception or design of the work, Critical revision of the article
7) Edward D. Levin - Conception or design of the work, Data analysis and interpretation, Drafting the article, Final approval of the version to be published

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