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Paternal factors in neurodevelopmental toxicology: THC exposure of male rats causes long-lasting neurobehavioral effects in their offspring

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ABSTRACT

The potential health risks of cannabis are of growing concern, including effects on reproduction and development. Extensive research has investigated risks associated with maternal exposure to THC during gestation and its impacts on the development of offspring, but little research has been done regarding paternal THC exposure effects prior to conception. We have previously found that paternal THC exposure in rats causes changes in sperm methylation. In an initial study we also showed that a 12-day paternal THC exposure prior to conception alters locomotor activity and impairs cognitive function of their offspring. This study investigated the cross-generational effects of chronic paternal THC exposure in rats (0, 2, or 4 mg/kg/day SC for 28 days) prior to mating with drug naïve females. The offspring of THC-exposed male rats had significant alterations in locomotor activity and cognitive function. Specifically, during adolescence there was significant locomotor hyperactivity in the offspring of males exposed to 2 mg/kg/day of THC. During the novel object recognition task, the controls maintained their relative preference for the novel object across the duration of the ten-min session while the rats whose fathers received THC (2 mg/kg/day) showed a significantly greater drop-off in interest in the novel object during the second half of the session. Learning in the radial-arm maze was significantly delayed in the offspring of males exposed to 4 mg/kg/day of THC. This study shows that premating chronic paternal THC exposure at multiple dose regimens can cause long-lasting detrimental behavioral effects in their offspring, including abnormal locomotor activity and impaired cognitive function. Future studies should investigate the underlying mechanisms driving these aberrant developmental outcomes and seek to identify possible treatments of alleviation in the presence of paternal THC exposure.

1. Introduction

The use of cannabis as a medical and recreational drug is becoming more prominent throughout the world and has led to many concerns about its potential adverse effects including impairments of brain development and behavioral function after parental cannabis use (Rey et al., 2002; Volkow et al., 2016). These concerns are well-warranted, as cannabis is known to cause acute deficits in learning, attention, and memory in users (Crane et al., 2013; Crean et al., 2011). The endocannabinoid system also plays a critical role in many neurodevelopmental processes, including early neural stem cell survival, neuronal connectivity, and synaptic function (Lubman et al., 2015). Effects of maternal cannabis or THC exposure during gestation has been widely studied, but until recently the effects on paternal exposure prior to conception has not received much attention. Combined paternal and maternal THC exposure has been shown to produce persisting neural and behavioral alterations in the offspring (Szutorisz et al., 2014a). More recently, we have found that paternal-only THC exposure also causes persisting behavioral effects in their offspring (Levin et al., 2019). These long-lasting cross-generational behavioral effects may be related to the THC-induced significant alterations in methylation of rat sperm DNA (Levin et al., 2019). In a companion study, we also found that cannabis exposure significantly alters sperm DNA methylation in humans (Levin et al., 2019).

Rates of cannabis use among pregnant women are increasing rapidly, just as fast as they are among non-pregnant woman of reproductive age (Brown et al., 2017). Accordingly, an extensive amount of research has been dedicated to investigating risks associated with maternal exposure to cannabis and THC during gestation to determine if there are long-term consequences to offspring health and

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neurodevelopment (El Marroun et al., 2018). One study in mice found that intraperinatal exposure to low doses of THC during pregnancy and prenatal development has negative impacts on cortical development of the offspring, and this is attributed to a disruption of spatiotemporal cannabinoid receptor signaling during the embryonic period (de Salas-Quiroga et al., 2015). Another study showed that prenatal exposure to cannabis in children was significantly related to increased impulsivity and hyperactivity, along with attention deficits (Goldschmidt et al., 2000). A prevailing hypothesis among researchers is that introduction to cannabis in the immature brain may lead to a frontal cortex that develops and functions aberrantly in adolescence; structural and functional neuroimaging studies support these claims (El Marroun et al., 2016; Smith et al., 2016).

Although maternal use of cannabis during pregnancy has been well-established as a substrate to future behavioral and cognitive issues in offspring, effects of paternal use prior to conception has received relatively little attention. It is now known that epigenetic modifications occur in the DNA of all cells, including sperm, allowing for chemical exposure to affect the development of future offspring through the paternal line (Soubry et al., 2014; Tang et al., 2015). Studies have already documented alterations in the behavior of offspring after paternal exposure to alcohol, nicotine, and opiates in rodent models (Goldberg and Gould, 2019). It follows suit that paternal cannabis exposure may also lead to developmental changes in offspring that would not normally occur, possibly leading to alterations in normal behavior. In recent studies, we found that 2 mg/kg/day of THC administered to adult male rats via oral gavage for 12 days significantly altered methylation of sperm DNA (Murphy et al., 2018a). Following this, we performed a separate study to determine if acute paternal THC exposure preconception could affect neurobehavioral function of the offspring (Levin et al., 2019). We found that this relatively brief exposure led to significant increases in habituation of locomotor activity and caused long-lasting attentional impairments in offspring relative to controls, suggesting THC can produce intergeneration effects.

However, there was a caveat in that study. The vehicle for THC used in our previous study contained 10 % ethanol. The vehicle controls also received 10 % ethanol but, it was possible that there may have been a unique interaction of paternal ethanol and THC on behavioral function in the offspring. Although the ethanol delivered was minimal, administration of alcohol and THC in other studies have been shown to exhibit synergy to impair object recognition, and it is possible effects could extend to other behavioral tests (Ciccocioppo et al., 2002). In the current study we explored the chronic effects of THC, rather than acute. Therefore, we conducted a study with an additional dose over a longer period of time without an ethanol vehicle. We exposed young adult male rats to 0, 2 or 4 mg/kg/day subcutaneously (sc) of THC for 28 days, and the vehicle for all groups was 5 % Tween80 in saline with no ethanol. Then we assessed the effects of this exposure on reproduction and neurobehavioral development of the offspring. A duration of 28 days was used to extend exposure into the second sperm cycle of Sprague-Dawley rats, which are commonly used to explore the effects of reproductive toxins (Sokol et al., 1999). Behavioral function was measured in a test battery including tests of locomotor activity, emotional function and cognition.

2. Methods

2.1. Design

The intergenerational effects of paternal THC exposure were investigated by dosing young adult Sprague-Dawley rats with 0, 2, or 4 mg/kg/day of THC via subcutaneous injection for 28 days. Controls received the vehicle containing saline and 5 % Tween80. Two days after the exposure ended, the male rats were then bred with drug-naive females. At weaning, 1 male and 1 female were chosen from each litter in each treatment group for behavioral testing. They were then tested on a battery of behavioral tests to assess locomotion, emotional function and cognition. Subjects were maintained on a reversed 12/12 day-night cycle and had ad libitum access to food and water, unless stated otherwise. All testing occurred under low, ambient light conditions during the animal's dark phase (between 8:00–17:00). A dim white light was used for to ensure visibility during testing. To minimize disturbance of the animals' circadian cycle the light was encapsulated with a metal housing and only the top portion of the light was exposed to direct light toward the roof of the testing chamber. All study protocols were approved by the Institutional Animal Care and Use Committee at Duke University and conducted in accordance with federal guidelines.

2.2. Paternal THC exposure

Nine-week-old, sexually mature male Sprague Dawley rats were housed 2–3 per cage and were dosed daily for 28 days via subcutaneous injection. There were three treatment groups: controls (N = 12) with vehicle only (5 % Tween80 in saline, mg/kg/day), THC-exposed rats (N = 10) receiving 2 mg/kg THC (Sigma-Aldrich St Louis, MO, USA) with 5 % Tween80 in saline, and THC-exposed rats (N = 10) receiving 4 mg/kg THC and 5 % Tween80 in saline. The 2 mg/kg THC dose was selected because it modeled human moderate daily cannabis use (Harte and Dow-Edwards, 2010a, b; Irimia et al., 2015; Rubino et al., 2009). Many people self-administer cannabis by smoking, but this was not chosen as a route for the current study because chronic smoke exposure is stressful for rats and the dose administered is difficult to control. The males were group housed 2–3/cage with all animals housed with others of the same treatment group. The sequence of paternal THC exposure and offspring behavioral testing is displayed in Table 1.

2.3. Mating

Two days after the end of THC exposure the male rats were mated to drug naïve females. Each THC-exposed and control male was housed together with a drug-naive young adult female Sprague-Dawley rat for five days. The dams were housed singly with their litters. Weaning occurred on day 21 after birth. Then the offspring were housed in same-sexed groups.

Table 1
Paternal THC Exposure and Behavioral Testing of Offspring.

Paternal THC (0, 2 or 4 mg/kg/day)	Behavioral Testing of Offspring					
	Postnatal Weeks					
For 28 days Ending 2 days Before Mating	4 Elevated Plus Maze	5 Figure-8 Locomotor Activity Test	6 Novelty Suppressed Feeding	7 Novel Object Recognition	11-Aug Radial Arm Maze	Dec-40 Operant Visual Attention Task

2.4. Behavioral testing of the offspring

Behavioral assessment began during adolescence and continued into adulthood with a battery of tests to index long-term effects of paternal THC exposure on offspring locomotor activity, cognition and emotional response during the latter stages of development into adulthood. There were 12 control litters and 10 litters each with THC 2 and 4 mg/kg/day exposed fathers. One male and one female from each litter were tested on the following behavioral test battery. This test battery covers a variety of cognitive, motor and emotional functions. It has been found to be sensitive to the effects of a variety of developmental toxicant exposures (Levin et al., 2010b, a; Roegge et al., 2008; Timofeeva et al., 2008a, b). The behavioral test methods given below were greatly similar to the methods used in our previous studies.

2.4.1. Week 4: elevated plus maze

The rats were tested on the elevated plus maze (Med Associates, St Albans, VT, USA) to assess their anxiety-like behavior vs. risk-taking behavior. The maze measured 142-cm × 104-cm × 76-cm high and consisted of two arms with 15-cm high, enclosed walls and two open arms with 2-cm railings. Each rat was assessed individually on the elevated plus maze for a single five-min session. The percentage of time the rat spent in the open vs. enclosed arms of the maze was calculated as an index of anxiety vs. risk taking. Also, the number of crossings across the center was counted as a measure of activity. The dependent measures were percent of time in the open arms to index anxiety-like behavior and the number of center crossings to measure locomotion in this five-min test.

2.4.2. Week 5: figure-8 locomotor activity test

Locomotor activity and its habituation were assessed in an enclosed maze in the shape of a figure-8 with two side alleys. The Figure-8 apparatus had a continuous alley measuring 10-cm x 10-cm, with the entire maze measuring 70-cm × 42-cm. Animals were permitted to freely explore the apparatus. Locomotor activity was indexed by the crossing of eight photo-beams located at approximately equal points throughout the alley. Photobeam breaks were tallied in 5-min blocks across the one-hour test session. The mean number of photobeam breaks per five-min block within the session indexed locomotor activity. The linear trend of decreasing beam breaks over the twelve sequential time blocks within the session indexed the habituation of activity with experience in the apparatus over the one-hour session. The log of the activity measure (photobeam breaks per 5 min block) was used to normalize the distribution.

2.4.3. Week 6: novelty suppressed feeding

To assess fear responsivity, the offspring rats were tested for the suppression of feeding behavior in a novel environment. Each rat had food restricted for 24-h prior to the test session. The novel environment consisted of a plastic rectangular cage (different from the home cage) placed in the middle of low light testing room, with no cage top and no bedding in the cage. Twelve standard rat chow pellets were weighed before testing and were spread across the cage floor in 4 rows of 3 pellets each. The sessions lasted 10 min each. Eating was defined as the act of chewing the food and not merely sniffing, holding, or carrying the food around in the mouth. The food pellets, which remained after the test session, were weighed to determine the amount of food eaten. The dependent measures were amount of food eaten, latency to begin eating, the number of eating bouts and the duration of eating.

2.4.4. Week 7: novel object recognition

Recognition of a novel vs. familiar object was used to test attention and memory in a low-motivational state. Tests were conducted in opaque plastic enclosures measuring 70-cm x 41-cm x 33-cm. Objects consisted of plastic, glass, or ceramic material and were randomized for each animal. Animals were first habituated to the apparatus in two 10-min sessions over the course of two days. Testing began on day 3 with a 10-min familiarization session in which two identical objects (A/A) were placed in the cage for the animal to explore. The A/A session was then followed by a one-h period spent in the animal's home cage. The animal was then placed back in the enclosure with one object from the A/A session and with another, dissimilar, "novel" object (A/B session). A one hour delay was chosen based on our own previous experience and from studies completed by other researchers who have found that a 24 h delay can lead to a failure of the rats to discriminate the novel from familiar object, while a 1 h delay has been shown to lead to good memory and recall (Besheer et al., 2001; Prickaerts et al., 2005; Sambeth et al., 2007). Between sessions, the objects were wiped clean in order to avoid odor recognition cues by the rats. The test session lasted for ten min. Analysis considered the preference in the first and second halves of the familiarization and test sessions. Typically, the behavior during the first five-min block within the test session rats will clearly differentiate novelty between the two objects compared with the second five min of the test session. The time in seconds spent actively exploring each object was recorded during each five-min block during the ten-min familiarization/test sessions and used for analysis.

2.4.5. Week 8-11: radial-arm maze

Spatial learning and memory were tested in the 16-arm radial maze. The maze was made of wood black painted with a central platform (50-cm diameter) and 16 radiating arms, each 10-cm wide x 60-cm in length. A food cup was positioned 2 cm from the end of each arm. Visual cues (cardboard shapes) were on the walls of the testing room to facilitate spatial orientation. The rats were habituated in the maze for two 10-min sessions in which they were placed on the central platform inside a large, black, round, opaque cylinder, with half-pieces of sugar coated cereal (Froot Loops®; Kellogg's Inc, Battle Creek, MI, USA). For the test sessions, twelve of the arms were baited at the beginning of each session to test working memory performance and the other four arms were always left un-baited to test reference memory (Hall et al., 2016a, b). The baited arms of the maze for each rat remained constant throughout the entire series of testing sessions, but which arms were baited differed randomly between rats. Each trial began by placing the rat on the central platform inside the opaque cylinder for 10 s. Then the cylinder was lifted, and the rat was allowed to roam the maze freely. Each session lasted 10 min or until the rat had entered all twelve baited arms, whichever occurred first. Each rat was assessed for working and reference memory errors over 18 sessions. Working memory errors were counted as repeat entries into baited arms, and reference memory errors were counted as entries into the arms that were never baited. Duration of responding was calculated as the total session time divided by the number of arm entries. There was one session run per day. The dependent measures were the number of working and reference memory errors as well as response duration (seconds per arm entry).

2.4.6. Weeks 12–40: operant visual attention task

The attention test was conducted as described in detail previously (Hall et al., 2016a, b). There is a long period of training for it. All the rats began at the same age. Each rat was placed in an operant chamber and trained to press one of two retractable levers in response to a visual cue light that was illuminated for a duration of 500 ms. If the cue-light became illuminated ("signal" trial), the animal needed to press the lever designated as the "signal" lever to receive a 20 mg food pellet reward. If the cue-light was not illuminated ("blank" trial), the animal needed to press the opposite lever in the chamber to receive the reward. The position (left, right) of "signal" and "blank" levers was randomized among the rats. If the rat made no response within 5 s of insertion of the response levers into the chamber, both levers retracted and a response "failure" was recorded. There were equal numbers of "signal" and "blank" trials in each test session with a total of 240 trials. "Hit" responses were correct choices on the signal trials while "correct rejection" responses were correct choices on blank trials. Percent correct hit

and percent correct rejection per session were the dependent measures for response accuracy on this attention task. Analysis was conducted of the choice accuracy data including these factors as well as THC exposure and sex.

2.5. Data analysis

For each behavioral test, the data were evaluated by analysis of variance. Litter was the unit of variance. The between-litters factor was THC treatment. The within litter factor was sex. Within-subjects repeated factors were sessions and time blocks within session. Because each litter contributed one male and one female, sex was treated as a repeated measure within litter. For interactions at $p < 0.10$, we also examined whether lower-order main effects were detectable after subdivision of the interactive variables (Snedecor, 1967). The $p < 0.10$ criterion for interaction terms was not used to assign significance to the effects, but rather to identify interactive variables requiring subdivision for lower-order tests of the main effects of THC, the variable of chief interest. A cut-off of $p < 0.05$ (two-tailed) was used as the threshold for statistical significance. The sample sizes needed to detect biologically important effects of 10–12 litters per treatment group were determined from prior developmental neurotoxicology studies.

3. Results

3.1. Physical development of THC exposed males

No significant effects were seen with the clinical health and body weight of the THC exposed male rats compared with controls.

3.2. Clinical signs of health during and after gestation

No significant effects were seen with litter size, sex ratio, birthweight or subsequent growth (Table 2). These moderate doses of 2 or 4 mg/kg/day THC administered to male rats during 28 days did not significantly affect rates of conception or birth indices.

3.3. Elevated plus maze test of anxiety-like behavior

There was no significant effect of paternal THC exposure to either 2 or 4 mg/kg/day on offspring behavior in the elevated plus maze (Table 3), either with percent open arm time (measure of anxiety, lower scores indicate greater anxiety) or center crosses (short-term locomotor activity during the five-min session). There was a significant main effect of sex on percent open arm time ($F(1,30) = 9.20$, $p < 0.01$) with males (33.2 ± 3.8 %) having less open arm time than females (43.4 ± 3.7 %).

3.4. Figure-8 apparatus locomotor activity test

This test of locomotor activity and its habituation in a one-h session was run twice, once during adolescence and again in young adulthood. The data were log transformed for analysis to decrease skewedness in variance. During adolescence, paternal THC exposure caused significant ($F(2,29) = 3.37$, $p < 0.05$) hyperactivity. The *post hoc* Dunnett's tests comparing the paternal THC groups to controls showed that the adolescent offspring of the 2 mg/kg/day paternal THC group had

Table 3
Elevated Plus Maze Measurements.

	Percent Open Arm Time	Center Crosses		Female
	Male	Female	Male	
Control	31.1 ± 6.1	36.5 ± 4.6	2.83 ± 0.53	2.67 ± 0.66
THC 2	35.6 ± 7.8	48.9 ± 7.0	2.73 ± 0.65	3.18 ± 0.46
THC 4	32.9 ± 6.3	45.6 ± 8.0	3.20 ± 0.77	2.60 ± 0.69

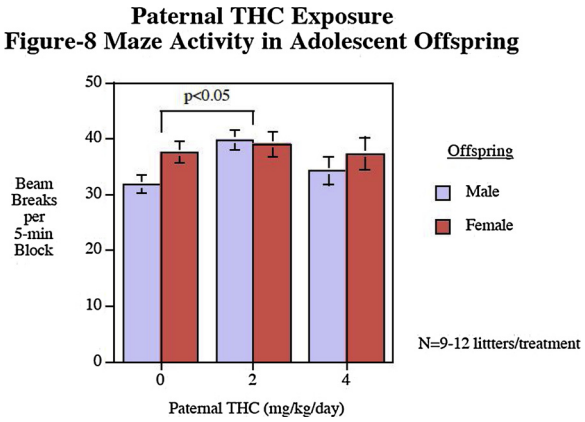

Fig. 1. Paternal THC exposure to 2 mg/kg/day for 28 days prior to mating caused significant ($p < 0.05$) locomotor activity of offspring during adolescence in the Figure-8 apparatus (mean ± sem).

significant ($p < 0.05$) hyperactivity relative to controls (Fig. 1). In adulthood, this paternal THC induced locomotor hyperactivity was no longer evident as a main effect, but there was a significant interaction of paternal THC treatment x session block ($F(22,319) = 1.70$, $p < 0.05$), but none of the Dunnett's test comparisons of the offspring of THC exposed fathers was significantly different from controls for any of the individual time blocks within the session. No significant sex differences were seen on the Figure-8 test during adolescence, but there was a significant main effect of sex ($F(1,18) = 14.50$, $p < 0.005$) in the test taken during adulthood. The males (40.9 ± 4.3) were significantly slower than the females (51.5 ± 4.5). Sex was not seen to significantly interact with paternal THC treatment at either age.

3.5. Novelty suppressed feeding test of fear response

There were no significant effects of paternal THC on the novelty suppressed feeding test (Table 4). No sex differences or sex interactions with paternal treatment were seen.

3.6. Novel object recognition test of non-spatial memory

In this test of non-spatial memory there was a significant ($F(1,26) = 6.15$, $p < 0.025$) main effect of the rats investigating the novel object more than the familiar object (Table 5). This demonstrates that the test was operating as intended. Analysis of the novelty preference showed that there was a significant paternal THC x time block interaction (F

Table 2
Developmental Health Measurements.

%Pregnant	Litter size	%Male	Birth Weight (g)		Weaning Weight (g)		
			Male	Female	Male	Female	
Control	100 %	12.3 ± 0.5	56.6 ± 5.1 %	8.4 ± 0.4	7.8 ± 0.3	55.2 ± 2.3	53.4 ± 2.1
THC 2	100 %	13.2 ± 0.7	51.6 ± 3.0 %	7.9 ± 0.2	7.5 ± 0.2	53.9 ± 2.2	51.8 ± 2.0
THC 4	83.30 %	11.9 ± 0.9	47.0 ± 3.7 %	8.6 ± 0.3	8.3 ± 0.3	58.6 ± 2.8	56.6 ± 2.8

Table 4
Novelty Suppressed Feeding Measurements.

	Latency to Eat (s)	Feeding Bouts	Feeding Duration (s)	Amount eaten (g)	
Control	123.4 ± 15.0	15.3 ± 1.5		142.4 ± 1.5	1.12 ± 0.23
THC 2	92.5 ± 11.8	18.2 ± 2.7		140.1 ± 6.5	0.91 ± 0.15
THC 4	111.0 ± 14.4	14.7 ± 2.1		138.8 ± 21.3	1.10 ± 0.16

Table 5
Novel Object Recognition Investigation Time.

	Min 1-5		Min 6-10	
	Familiar	Novel	Familiar	Novel
Control	27.6 ± 4.7	34.8 ± 4.7	15.6 ± 2.3	22.5 ± 3.3
THC 2	25.3 ± 4.6	36.7 ± 5.7	23.9 ± 6.7	16.5 ± 3.8
THC 4	22.6 ± 4.8	34.4 ± 6.3	18.4 ± 3.8	17.2 ± 3.7

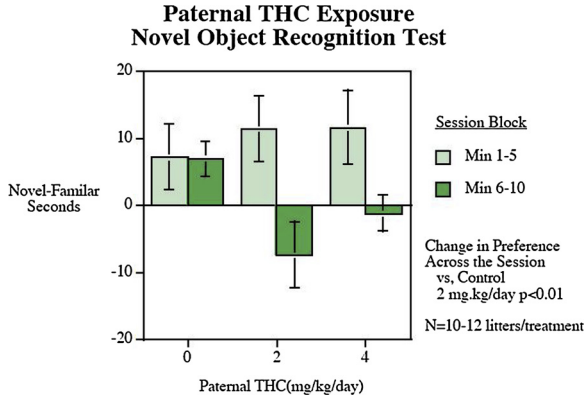

Fig. 2. Paternal THC exposure to 28 days of THC at 2 mg/kg/day of THC caused a significantly ($p < 0.01$) greater loss in novel object preference relative to controls (mean ± sem).

(2,30) = 5.94, $p < 0.01$). Assessment of the change in novelty preference across the test session showed a significant main effect of THC treatment ($F(2,30) = 5.94$, $p < 0.01$). The *post hoc* Dunnett's test comparing the treated vs control group that the offspring of the paternal 2 mg/kg/day THC exposure group had significantly ($p < 0.01$) greater drop-off in novelty preference compared with controls (Fig. 2). The offspring of the paternal 4 mg/kg/day group showed a similar mean difference but this was not quite significantly different from control. There were no significant sex differences on the novel object test.

3.7. 16-Arm radial maze test of spatial learning and memory

In this test of spatial learning and memory there was a significant impairment caused by paternal THC exposure, yet no sex differences were observed. In the overall analysis of working memory errors there was an interaction of paternal THC treatment x session block ($F(6,69) = 1.98$, $p < 0.09$) that promoted follow-up with the tests of paternal THC effects on trends of improvement over session blocks. There was a significant ($F(2,23) = 3.79$, $p < 0.05$) effect of paternal THC exposure on the improvement in working memory errors over the course of the four three-session blocks of training. The *post hoc* Dunnett's tests showed that the 4 mg/kg/day group showed a significantly ($p < 0.05$) lower trend of errors over session blocks than controls. As shown in Fig. 3, the offspring of males exposed to 4 mg/kg had slower acquisition than controls. No significant paternal THC effects were seen with reference memory errors.

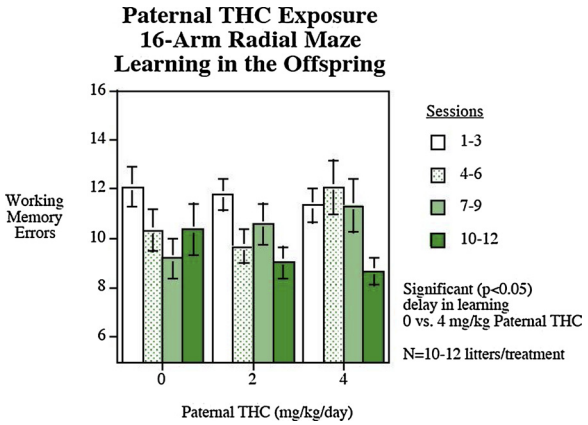

Fig. 3. Working memory errors on the 16-arm radial maze (mean ± sem). The offspring of the males exposed to 4 mg/kg/day had a significantly ($p < 0.05$) slower acquisition as indicated by a greater quadratic trend relative to control.

3.8. Operant visual signal detection test of learning and attention

In this operant visual signal detection test of attentional function the interaction of paternal THC treatment and error type ($F(2,29) = 2.86$, $p < 0.08$) prompted further analysis of the simple main effects of paternal THC within each trial type (percent hit and percent correct rejection). As shown in Fig. 4, paternal THC treatment did not significantly affect either percent hit (Control = 83.0 ± 1.3 %; THC 2 mg/kg/day = 80.7 ± 1.5 % and THC 4 mg/kg/day = 79.4 ± 1.7), or percent correct rejection (Control = 89.5 ± 1.2 %; THC 2 mg/kg/day = 92.7 ± 0.6 % and THC 4 mg/kg/day = 90.5 ± 1.1). There was a significant main effect of session with improvement with continued training ($F(5,145) = 77.00$, $p < 0.0005$) showing that the test successfully indexed learning of the task. There was a significant ($F(1,29) = 90.4$, $p < 0.0005$) effect of error type (hit vs. correct rejection) an effect that is always seen with this task. There were no interactions evident for paternal THC interacting with either of these factors or with

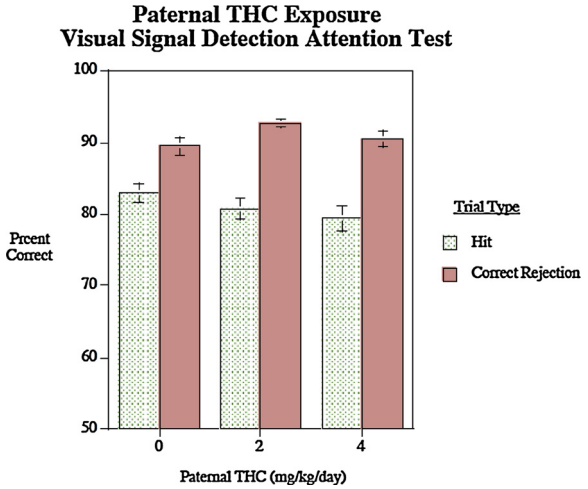

Fig. 4. Percent correct performance for hit and correct rejection trials in the visual signal detection attention test (mean ± sem).

sex which did not itself have a significant effect.

4. Discussion

The current study investigated the intergeneration effects of chronic THC exposure of young adult male rats prior to mating with drug naïve female rats. Twenty-eight days of THC exposure in rats at a dose that modeled moderate-high cannabis use (2 or 4 mg/kg/day, SC) produced significant behavioral effects in their offspring relative to the offspring of males exposed to the vehicle. Although the clinical health of the offspring was not significantly affected by paternal exposure, these rats did show alterations in attention, memory, and locomotor activity when compared to controls. Specifically, during adolescence there was significant locomotor hyperactivity in the offspring of rats exposed to 2 mg/kg/day of THC for 28 days prior to mating. This pattern of hyperactivity did not persist into adulthood. During the novel-object recognition task, the controls maintained their relative preference for the novel object across the duration of the ten-min session while the rats whose fathers received THC (2 mg/kg/day) showed a significantly greater drop-off in interest in the novel object during the second half of the session. Although the NOR task is not designed to measure attention, it has been suggested that duration and frequency of object exploration in the familiarization session of the NOR test is a valid indicator of attention (Piper et al., 2005). This follows suit with researchers who have studied overt attention in humans, which is measured by the number of eye fixations on an object (Kim et al., 2013; Riggs et al., 2011). Specifically, if an animal pays more attention to the objects provided in the familiarization phase they are more likely to encode and retain the memories of these objects in long-term memory, which should lead to the animal spending more time interacting with the novel object in later phases. Following this, rats in the THC (2 mg/kg/day) group may be exhibiting a reduced ability to switch and hold attention on the novel object in the test phase.

The present findings are generally in agreement with the existing literature on paternal preconception exposure to drugs of abuse. As with other drugs of abuse (Goldberg and Gould, 2019), paternal THC exposure led to highly specific alterations in behavior which affected select assays while sparing general offspring health and unrelated behaviors. These data are also complementary to studies that have investigated cannabinoid effects on neurophysiology. For example, it is known that the medial prefrontal cortex (mPFC) plays a vital role in attention, memory, and motivational processes by receiving inputs from the nucleus accumbens (NAc), basolateral amygdala, and ventral hippocampus (Britt et al., 2012; Castellanos et al., 2002). A recent optogenetic study which used intraperitoneal injections of THC (5 mg/kg/day, 14 days) in rats found that chronic THC disrupts the balance of glutamatergic corticolimbic input to the NAc, weakening mPFC inputs and strengthening those from the amygdala and hippocampus (Hwang and Lupica, 2020). The shift in modulatory strength of the NAc from the prefrontal areas to limbic sites provides a neurochemical foundation for the ability of THC to cause specific behavioral changes and may help to explain why offspring in the current study showed issues of executive function, namely attention and memory. Interestingly, disruption of these circuits have also been linked to behavioral disinhibition and impulsivity, along with being implicated in the pathophysiology of attention deficit hyperactivity disorder (ADHD) (Plessen et al., 2006; Schoenbaum et al., 2003; Winstanley et al., 2004).

This study shows that premating chronic paternal THC exposure at multiple dose regimens can cause detrimental behavioral effects, including abnormal locomotor activity, attentional impairment, and reduced memory and motivation. Attentional deficits are characteristic symptoms of ADHD, and as such, are highly comorbid with a wide range of psychiatric and behavioral disorders (e.g. (Solberg et al., 2018). In general, attention deficits tend not to have known causes, with the exception of toxicant-induced deficits such as fetal alcohol or tobacco exposure (Kingdon et al., 2016; Pagani, 2014a, b). The present rodent data suggest that paternal epigenetic effects may represent an additional candidate cause of attentional dysfunction, as preconception THC exposure led to an inattentive phenotype in the absence of other risk factors. Further, these data indicate that THC is a compound of particular interest in this area and requires further investigation.

The neurobehavioral effects seen in the offspring of male rats exposed to THC prior to mating may have been caused by abnormal DNA methylation patterns in the sperm (Murphy et al., 2018a, b). In our previous article we reported on the character of this THC-induced abnormal methylation (Murphy et al., 2018a, b). In that study, we evaluated associations between cannabis/THC exposure and altered DNA methylation in sperm from humans and rats. DNA methylation, measured by reduced representation bisulfite sequencing, differed in the sperm of human users from non-users by at least 10 % at 3979 CpG sites. Pathway analyses indicated Hippo Signaling and Pathways in Cancer development as enriched with altered genes. These same two pathways were also enriched with genes having altered methylation in sperm from THC-exposed versus vehicle-exposed rats $p < 0.01$. Data validity is supported by significant correlations between THC exposure levels in humans and methylation for 177 genes, and substantial overlap in THC target genes in rat sperm in this study and genes previously reported as having altered methylation in the brain of rat offspring born to parents both exposed to THC during adolescence Szutorisz et al., 2014a, b). In humans, cannabis use was also associated with significantly lower sperm concentration (Murphy et al., 2018a, b). Most of the methylation marks are removed upon fertilization, although there are some which remain and are passed on to the offspring (Tang et al., 2015).

It has long been known that maternal exposure after conception to a wide variety of environmental chemicals, therapeutic drugs and drugs of abuse including cannabis can cause neurobehavioral impairments in the offspring. This study and others are contributing to a quickly emerging literature that paternal chemical exposure before conception can produce behavioral alterations in the offspring that persist into adulthood. These studies may also provide insight into non-monotonic effects seen with certain toxins. The example from this study being that the 2 mg/kg/day exposure often led to significant, deleterious outcomes while the higher dose of 4 mg/kg/day did not always produce significant effects. It is possible that this dosage increase may meet thresholds for additional neurotoxic or compensatory effects on biological machinery, which could in turn attenuate behaviors like hyperactivity. Also, although significant behavioral effects were not severely detrimental to the behavior or health of the offspring, and in some cases dissipated in adulthood, these findings are crucial when discussing reproductive toxins. The adolescent brain is a sensitive organ that is subject to countless psychological, neurochemical, and social changes. Any drug that can produce effects through maternal or paternal lineage that differ from the norm should be carefully regulated to ensure that humans do not succumb to the same or similar adverse effects.

Credit author statement

Zade R. Holloway designed the study, analyzed the results, dosed the subjects and wrote the article.

Andrew B. Hawkey designed the study, dosed the subjects and wrote the article.

Erica Pippin dosed and tested the subjects and helped write the article.

Hannah White dosed and tested the subjects and helped write the article.

Corinne Wells dosed and tested the subjects and helped write the article.

Bruny Kenou dosed and tested the subjects and helped write the article.

Amir H. Rezvani designed the study and helped write the article.

Susan K. Murphy designed the study and helped write the article.

Edward D. Levin designed the study, analyzed the results and wrote the article.

Declaration of Competing Interest

There are none.

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