

Paternal cannabis extract exposure in rats: Preconception timing effects on neurodevelopmental behavior in offspring

Zade R. Holloway^a, Andrew B. Hawkey^a, Alexandra K. Torres^a, Janequia Evans^a, Erica Phippen^a, Hannah White^a, Vaishnavi Katragadda^a, Bruny Kenou^a, Corinne Wells^a, Susan K. Murphy^b, Amir H. Rezvani^a, Edward D. Levin^{a,*}

^a Department of Psychiatry and Behavioral Sciences, Duke University Medical Center, Durham, NC, USA

^b Department of Obstetrics and Gynecology, Duke University Medical Center, Durham, NC, USA

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ABSTRACT

Maternal toxicant exposure during gestation can have deleterious effects on neurobehavioral development of the offspring. The potential risks engendered by paternal toxicant exposure prior to conception have been largely understudied. Recently, we found that chronic THC exposure prior to conception in male rats causes long-lasting behavioral impairment in their offspring. The current study examined the effects of chronic preconception exposure to cannabis smoke extract in Sprague-Dawley rats at two different phases in sperm development. One group received daily subcutaneous (sc) injections of THC in cannabis extract at 4 mg/kg/day for 28 days until three days prior to mating with untreated females (late exposure group). Another group received the same regimen except they underwent 56 days of drug abstinence prior to mating (early exposure group). These were compared with a control group treated with vehicle. The offspring underwent a battery of tests for behavioral function to assess motor, emotional and cognitive function. On the elevated plus maze test, the offspring of both paternal cannabis smoke extract (CSE) exposure groups had significantly more time on the open arms than control offspring, indicative of greater risk-taking behavior. No significant main effects of CSE exposure were seen on adolescent or adult locomotor activity in the figure-8 apparatus. In the novel object recognition test, there was a significantly greater drop-off in novel object preference across the session in the male, but not female offspring of the late exposure group. There was also a sex-selective effect of paternal CSE treatment in the 16-arm radial maze test of memory function. Female offspring of the late exposure group had significantly more working memory errors than control females in the first half of the 12-session training sequence. No significant effects were seen in the operant visual signal sustained detection test of attention. This study shows that there are long-lasting behavioral consequences of preconception CSE exposure through the paternal lineage in rats.

1. Introduction

Cannabis use as a recreational and medical substance is becoming more prominent and its effects on brain development, behavior, and health are gaining much-needed attention in research (Karila et al., 2014; Volkow et al., 2016). Cannabis use has a variety of psychological effects and can cause acute deficits in learning, attention and memory in users. Maternal exposure during pregnancy can also lead to adverse effects in the offspring, as the endocannabinoid system plays a critical role in development, including early neural stem cell survival, neuronal connectivity, and synaptic function (Crane et al., 2013; Crean et al., 2011; Lubman et al., 2015). Paternal use of cannabis can also adversely

affect development of his offspring, but these effects have been greatly understudied.

With the emerging understanding of the scope of epigenetic modifications present in sperm, and their vulnerability to environmental effects, it is becoming increasingly recognized that there is potential for altered epigenetic programming in sperm cells to be carried forward through conception and affect the development of future offspring through the paternal line (Anderson et al., 2014; Soubry et al., 2014). For example, paternal THC exposure in male rats for 28 days prior to mating causes impairments in locomotor activity, attention, and reduced memory and motivation of the offspring (Holloway et al., 2020). A shorter, sub-chronic exposure of THC for 12 days in male rats

* Corresponding author at: Department of Psychiatry and Behavioral Sciences, Duke University Medical Center, Box 104790 DUMC, Durham, NC 27710, USA.
E-mail address: edlevin@duke.edu (E.D. Levin).

led to fewer, but meaningful effects in the offspring, including significant increases in habituation of locomotor activity and caused long-lasting attentional impairments relative to controls, further validating that THC can produce intergenerational developmental effects (Levin et al., 2019). Others have suggested that some deficits caused by paternal germline THC exposure may arise from dysregulation of genes in critical brain areas, such as striatal genes related to synaptic plasticity in male and female offspring (Szutorisz et al., 2016).

The heritability of behavioral dysfunction from paternal drug use is likely driven, at least in part, by abnormal methylation of the sperm genome, as we have observed in both human males with cannabis exposure and male rats with analogous levels of THC exposure (Murphy et al., 2018). Among the alterations observed, hypomethylation was seen in Discs-Large Associated Protein 2 (*DLGAP2*), a protein involved in synapse organization and neuronal signaling (Schrott et al. 2020). When dysregulated, this protein has been associated with various neurological disorders, such as autism spectrum disorder (ASD), post-traumatic stress disorder (PTSD), and schizophrenia (Chertkow-Deutsher et al., 2010; Jiang-Xie et al., 2014; Li et al., 2014). What remains less understood is whether the maturity of the sperm at the time of exposure influences the likelihood or heritability of such genetic and behavioral abnormalities.

The current study was conducted to determine the behavioral effects of paternal exposure to the full complement of cannabis constituents including THC, observing the differences in cannabis smoke extract (CSE) effects during a period of sperm maturity, just prior to conception, and those produced by exposure to immature sperm, followed by an extended abstinence. In our previous studies, we examined the effects of THC at 2 mg/kg/day orally and through subcutaneous injection, because it modeled moderate daily cannabis use, and 4 mg/kg/day subcutaneous to model high daily cannabis use (Harte and Dow-Edwards, 2010; Holloway et al., 2020; Irimia et al., 2015; Levin et al., 2019; Rubino et al., 2009). The subcutaneous method was chosen over oral administration to better model chronic rather than acute cannabis use, and also to avoid any carryover effects the 10 % ethanol vehicle used in the oral study may have had. The dose of THC in the cannabis smoke extract used was kept at 4 mg/kg/day for 28 days, the same as in our previous study to facilitate comparisons.

To assess the effects of sperm maturity on heritable behavioral effects, we tested the effects of 28 days of THC in cannabis smoke extract exposure ending either 3 days or 56-days prior to mating. This was done to determine whether avoiding CSE exposure during the major portions of the sperm generation cycle in the male rats would prevent damage to the neurodevelopment of the offspring. Twenty-eight days reflects the time period of two seminiferous epithelium cycles, while 56 days reflects the full length of the rat spermatogenic cycle; in contrast a full cycle takes 74 days in humans (Amann, 2008; Kolasa et al., 2004). In one sense, the CSE exposure during the earlier phase of the spermatogenic cycle tested the possibility of “recovery”, with an eight-week period of drug abstinence between the end of chronic CSE exposure and conception. In another sense, this group represented a test of the differential effect of CSE exposure during early and later stages of sperm development. The 28-day CSE exposure that ended shortly before mating would have resulted in sperm used for fertilization that were exposed during later maturation stages, but these same sperm would have not experienced the exposure during their earlier proliferative stages of maturation. In contrast, the CSE exposure for 28 days that ended 56 days prior to mating would have exposed progenitor cells including undifferentiated spermatogonia without added exposure during the later stages of sperm maturation (De Rooij, 2017). Although exposure does occur, this group should experience a full sperm cycle recovery throughout the 56 day period. Therefore, we hypothesize that offspring of the 56-day group will exhibit some form of recovery compared to offspring in the 28-day group.

2. Methods

2.1. Design

The generational effects of paternal CSE exposure across generations were investigated by dosing young adult Sprague-Dawley rats (Charles River, Raleigh, NC, USA) with 0 or 4 mg/kg/day of THC in cannabis smoke extract via subcutaneous injection for 28 consecutive days. The control group received the vehicle containing saline and 5% Tween80. Three days after the exposure ended, the male rats in the “late exposure group” were bred with 9-week old drug-naïve females. In the “early exposure group” there were 56 days with no cannabis smoke extract exposure between the end of the 28-day dosing regimen and mating. There were 11 litters per condition. At weaning, one male and one female were chosen from each litter in each treatment group for behavioral testing. The study was run in three cohorts with each condition in each cohort. The offspring were then evaluated using 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 to ensure visibility during testing. To minimize disturbance of the animals’ circadian cycle, lights were encapsulated with a metal housing and directed at either the ceiling or the roof of the testing chamber, as relevant. 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 cannabis smoke extract 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 with 11 litters produced with each condition: controls with vehicle only (5% Tween80 in saline), and experimental groups receiving cannabis smoke extract (NIDA Drug Supply Program, Research Triangle Institute International, Research Triangle Park, NC, USA) calculated to contain 4 mg/kg THC (NIDA, USA) in a 5% Tween80 solution. THC content was calculated based on the manufacturer’s certificate of analysis, of which the CSE extract contained 27.30 % delta-9-THC, 1.31 % cannabidiol, and 1.40 % cannabinol. Many people self-administer cannabis by smoking, but inhalation 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 and stressful for the rat relative to injection. The males were group housed 2-3/cage with all animals housed with others of the same treatment group, and were marked with different stripe marks on their tails for identification. The sequence of paternal CSE exposure and offspring behavioral testing is displayed in Table 1.

2.3. Mating and rearing

Either three or 56 days after the end of CSE exposure, the male rats were mated to drug naïve females. Each CSE-exposed and control male was housed together with a drug-naïve young adult female Sprague-Dawley rat for five days in a typical housing cage. Pregnancy was determined by weighing of the females throughout the five day period. The dams were housed singly with their litters. Weaning occurred on day 21 after birth. After weaning, the offspring were housed in same-sex, within-treatment groups with up to three rats per cage. Males were euthanized after pregnancy was determined and females were euthanized after weaning.

2.4. Behavioral testing of the offspring

Behavioral assessment began during adolescence and continued into

Table 1
Paternal CSE Exposure and Behavioral Testing of Offspring.

Paternal THC in CSE			Behavioral Testing of Offspring			
(0 or 4 mg/kg/day)			Postnatal Weeks			
For 28 days	4	5	6	7	8–11	12–40
Ending	Elevated	Figure-8	Novelty	Novel	Radial	Operant
56 or 3 days	Plus	Locomotor	Suppressed	Object	Arm	Visual
Before	Maze	Activity	Feeding	Recognition	Maze	Attention
Mating		Test				Task
		Also as adults				

adulthood with a battery of tests to index long-term effects of paternal cannabis smoke extract exposure on offspring locomotor activity, cognition and emotional response during the latter stages of development into adulthood. There were 11 control litters and 11 litters each from the early and late exposure groups. One male and one female from each litter were tested on the following behavioral test battery. This test battery begins one week following weaning (4 weeks of age) and covers a variety of cognitive, motor and emotional functions. This battery has been shown to be sensitive to the effects of a variety of developmental toxicant exposures (Levin et al., 2010; Roegge et al., 2008; Timofeeva et al., 2008a, b).

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 plexiglass maze measured 142-cm x 104-cm x 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 and recorded in real-time by a technician blind to the treatment. 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. Between sessions, the maze was disinfected and deodorized (RESCUE, Oakville, ON, Canada) in order to avoid odor recognition cues by the rats.

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 apparatuses each have a continuous metal alley measuring 10-cm x 10-cm, with the entire maze measuring 70-cm x 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. After each session the apparatus was disinfected and deodorized to avoid odor recognition cues by the rats.

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, but were allowed access to water. 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. The cage measured 36-cm x 25-cm x 19-cm. Twelve standard rat chow pellets (LabDiet,

Brentwood, MO, USA) weighing approximately 4.5 g per piece 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. All dependent measures were scored by a technician in real-time who was blinded to the treatment group. To address the issue of consistency with the rating system, inter-rater reliability was assessed for this behavioral measure. If a rat climbed out of the cage during testing, the timer was stopped and the rat was placed back in the cage to continue testing.

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 used during familiarization/test sessions were randomized and evenly balanced between novel vs. familiar objects for each animal within each treatment group, consisting of either plastic, glass, or ceramic material, and were heavy enough that the rats could not move them. Rats were considered “attending” to the object if they were looking at the object from a distance no more than an inch away, as well as if they were sniffing, climbing, or standing on the object. 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 (Hall et al., 2016). 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 observed during the first five-min block within the test session shows the 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 in real-time by a technician blind to the treatment during each five-min block during the ten-min familiarization/test sessions and used for analysis. To address the issue of consistency with the rating system, inter-rater reliability was assessed for this behavioral measure.

2.4.5. Week 8–11: radial-arm maze

Spatial learning and memory were tested in the 16-arm radial maze. One week before the beginning of training the rats were food restricted to approximately eighty-five percent of free-feed body weight with daily feeding. This was continued throughout radial-arm maze testing and the

following attention testing. The maze was made of wood painted black 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). The rats were fed daily after testing. During the habituation sessions rats were not allowed access to the arms of the maze. 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., 2016). 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 12 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). All data was recorded in real-time by a technician blind to the treatment, and the maze and food cups were disinfected and deodorized between experiments to avoid odor recognition cues by the rats. The 6-session block was used so as to not lose data from rats that did not complete the maze for individual sessions. When there were incomplete sessions, the mean of the remaining sessions of the block was taken. There was no difference by treatment for the number of incomplete sessions.

2.4.6. Weeks 12–40: operant visual attention task

The sustained attention test was conducted as described in detail previously (Hall et al., 2016). The rats undergo extensive training to criterion for this task and training data was not included in the final analysis for treatment effects. All the rats began at the same age and were food-restricted one week prior and throughout the task to maintain 85 % of free-feed body weight. Rats were fed all of their food at the end of the day. 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 (Bio-Serve, Flemington, NJ). If the centrally located 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. At the end of each trial, levers retracted and the cue light turned off to signal the end of a trial, and the entry of the levers again signaled the beginning of a new trial. There were equal numbers of "signal" and "blank" trials in each test session with a total of 240 trials, and "no response" trials were included. "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, and the "no response" trials were not factored in to these percentages. Analysis was conducted of the choice accuracy data including these factors as well as cannabis smoke extract exposure and sex.

2.5. Data analysis

For each behavioral test, the data were evaluated by a multilevel analysis of variance (SuperAnova, SAS, Cary, NC, USA). Each behavioral test had embedded assessments of its validity. Litter was the unit of variance. The principal between-litters factor was paternal cannabis smoke extract (CSE) treatment with three groups: vehicle-treated control, early CSE treatment (4 mg/kg/day for 28 days ending 56 days prior to mating) and late CSE treatment (4 mg/kg/day for 28 days ending three days prior to mating). The study was run in three cohorts with litters from sires from each of the treatment groups represented in each cohort. Cohort was included as a control factor in the analyses. Because each litter contributed one male and one female, sex was treated as a repeated measure within litter. Within-subjects repeated factors varied among the different tests with repeated sessions and time blocks within session. For interactions at $p < 0.10$, we also examined whether lower-order main effects were detectable after subdivision of the interactive variables (Snedecor and Cochran, 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 cannabis smoke extract, the variable of chief interest. A cut-off of $p < 0.05$ (two-tailed) was always used as the threshold for statistical significance in the final analysis. Dunnett's *post hoc* tests were used to compare each of the cannabis smoke extract exposed groups to control; this test was used to restrict the probability of reporting false positives.

3. Results

3.1. Physical health of cannabis smoke extract exposed males

The 4 mg/kg/day dose of THC in CSE given for 28 days did not significantly affect the health of males. They showed normal growth according to growth charts from the vendor, Charles Rivers labs.

Clinical Signs of Health of Dams and Offspring During and After Gestation

No effects of paternal treatment were found for measures of breeding success or pup health and growth (group means in Table 2). This analysis included pregnancy rate, litter size, male-female ratio, body weight at birth and at weaning, or ano-genital distance at birth or weaning. Ano-genital distance was measured with digital calipers, taking the distance from the anal cavity to the urethra while the pups were positioned on their back. As expected, a significant effect of sex was observed on ano-genital distance both at birth, ($F(1, 61) = 1399.56, p < 0.05$), and at weaning, ($F(1, 59) = 526.02, p < 0.05$).

3.2. Elevated plus maze test of anxiety-like behavior

On the elevated plus maze there was a significant ($F(2,24) = 4.42, p < 0.025$) main effect of paternal cannabis smoke extract exposure with percent time in the open arms regardless of sex. As shown in Fig. 1, Dunnett's *post-hoc* tests showed that the offspring of the early exposure group (48.9 ± 7.1), as well as the offspring of the late exposure group (47.9 ± 5.9) had significantly ($p < 0.05$) more time on the open arms than control offspring (35.0 ± 4.0), indicative of greater risk-taking behavior. There was also a significant ($F(2,24) = 5.65, p < 0.01$) effect of paternal cannabis smoke extract exposure on the number of center crosses in the elevated plus maze, a measure of locomotor activity. *Post-hoc* Dunnett's tests did not detect any significant differences between controls (3.7 ± 0.4) and either the early (3.1 ± 0.5) or late cannabis smoke extract (5.0 ± 0.5) paternal treatment groups (Table 3).

3.3. Figure-8 apparatus locomotor activity test

No significant main effects or interactions of paternal cannabis smoke extract exposure were seen on adolescent locomotor activity in

Table 2
Developmental Health Measurements.

	%Pregnant	Litter size	%Male	AGD (birth)		Birth Weight (g)		Weaning Weight (g)	
				Male	Female	Male	Female	Male	Female
Control	100	13.1 ± 0.9	49.1 ± 3.9	2.5 ± 0.07	0.5 ± 0.04	8.2 ± 0.3	8.0 ± 0.3	55.0 ± 2.0	53.5 ± 2.0
Early Exp	91.7	13.4 ± 0.7	42.5 ± 3.0	2.7 ± 0.07	0.6 ± 0.04	8.1 ± 0.3	7.7 ± 0.2	54.6 ± 1.8	52.8 ± 2.0
Late Exp	91.7	14.1 ± 0.7	46.2 ± 3.5	2.3 ± 0.10	0.6 ± 0.02	7.8 ± 0.2	7.7 ± 0.2	54.1 ± 1.8	52.2 ± 1.5

Paternal Cannabis Exposure Effects on Offspring Behavior
Elevated Plus Maze: Percent Open Arm Time

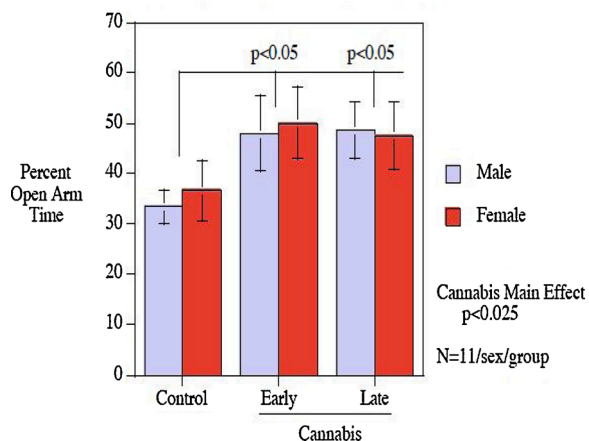


Fig. 1. Elevated Plus Maze, percent open arm time (mean ± sem).

Table 3
Elevated Plus Maze Measurements.

	Percent Open Arm Time		Center Crosses	
	Male	Female	Male	Female
Control	33.3±3.3	36.7±5.9	4.3±0.7	3.2±0.6
Early Exp*	47.9±7.6	50.0±7.1	2.3±0.5	3.9±0.8
Late Exp*	48.5±5.7	47.4±6.5	5.0±0.5	5.0±0.8

* Significantly higher than Control (p<0.05).

the figure-8 apparatus. There was a significant effect of sex (F(1,24) = 12.57, p < 0.005) with females being faster than males. Also, there was a pronounced habituation with lower activity across the session (F(11,264) = 89.43, p < 0.0005).

The offspring were retested in adulthood for locomotor effects and its habituation on the Figure-8 apparatus after being assessed on the attention test. No significant main effects of paternal cannabis smoke extract exposure or interaction with sex were seen. There was an interaction of paternal cannabis smoke extract x sex x session block (F(22,264) = 1.47, p < 0.09) which prompted follow-up of the simple main effects of paternal cannabis smoke extract on the linear trend of habituation across blocks for each sex. There were not significant effects of paternal cannabis smoke extract exposure on the linear rate of habituation across session blocks for either males or females.

3.4. Novelty suppressed feeding

No significant effects of paternal cannabis smoke extract exposure were seen on offspring novelty suppressed feeding in terms of latency to begin eating, duration of eating and the amount of food eaten. There was a significant effect of paternal cannabis smoke extract treatment on the number of bouts of eating (F(2,24) = 9.19, p < 0.005). Post-hoc Dunnett’s tests showed that the offspring of the early exposure group had significantly fewer eating bouts than controls (p < 0.01) (Table 4). There were significant main effects of sex for the amount of food eaten (F(1,24)

Table 4
Novelty Suppressed Feeding Measurements.

	Latency to Eat (s)	Feeding Duration (s)	Amount eaten (g)	Feeding Bouts
Control	104.5 ± 9.3	168.4 ± 12.9	1.64 ± 0.18	12.7 ± 0.7
Early Exp	127.5 ± 12.5	135.0 ± 14.8	1.23 ± 0.14	7.8 ± 0.6*
Late Exp	139.2 ± 23.4	132.4 ± 20.9	1.41 ± 0.18	10.4 ± 1.1

* Significantly lower than Control (p < 0.01).

= 7.93, p < 0.01, Males = 1.61 ± 0.13 g, Females = 1.24 ± 0.11 g) and the duration of feeding (F(1,24) = 29.25, p < 0.0005, Males = 168.2 ± 13.2 s, Females = 122.3 ± 8.8 s) with males having greater for both. The latency to begin feeding and feeding bouts did not show significant main effects of sex. None of the measures had interactions sex x treatment that would prompt follow-up analysis.

3.5. Novel object recognition test of non-spatial memory

There was an effect of paternal cannabis smoke extract on the novel object recognition test (Table 5). The overall analysis did not show a significant main effect of paternal cannabis smoke extract exposure but there was a higher level interaction involving paternal cannabis smoke extract exposure. The paternal cannabis smoke extract x sex x session block x novel vs. familiar object interaction (F(2,24) = 2.67, p < 0.09) called for tests of the simple main effects of paternal cannabis smoke extract exposure (Fig. 2). Follow-up tests of paternal cannabis smoke extract exposure effects on novel-familiar preference change across the session showed that the male offspring of the late exposure group had a significantly greater drop-off than controls (p < 0.025) while there was no significant effect with the early exposure group. No significant effects were seen in the females. Both males (p < 0.005) and females (p < 0.01) showed significant preferences for the novel object.

3.6. -Arm radial maze test of spatial learning and memory

Overall analysis of working and reference memory errors over the first and second halves of training detected interactions of paternal cannabis smoke extract treatment x sex (F(2,24) = 4.10, p < 0.05), paternal cannabis smoke extract x sex x session block (F(2,24) = 3.02, p < 0.07) and paternal cannabis smoke extract x sex x error type (F(2,24) = 3.94, p < 0.05). Follow-up tests of the simple main effects of paternal cannabis smoke extract were made at the highest-level interaction.

With working memory errors, there was a significant interaction of paternal cannabis smoke extract x sex x session block interaction (F(2,24) = 3.56, p < 0.05). Tests of the simple main effects of paternal cannabis smoke extract treatment on offspring working memory error

Table 5
Novel Object Recognition Investigation Time (s).

	Min 1–5		Min 6–10	
	Familiar	Novel	Familiar	Novel
Control	29.3 ± 3.1	53.2 ± 9.7	17.3 ± 2.5	23.5 ± 3.7
Early Exp	28.0 ± 4.2	41.3 ± 3.3	27.7 ± 2.9	27.2 ± 4.2
Late Exp	24.5 ± 42.3	51.2 ± 4.3	23.5 ± 3.7	24.9 ± 4.4

**Paternal Cannabis Exposure Effects on Offspring Behavior
Novel Object Recognition: Novel Object Preference Change**

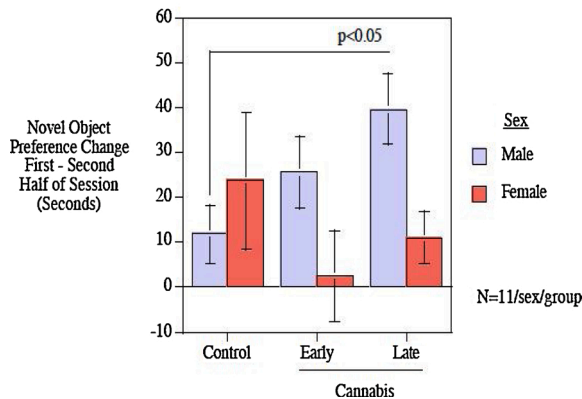


Fig. 2. Novel Object Recognition time spent investigating novel minus familiar object (s) change from the first to the second half of the session (mean ± sem).

rates in the radial-arm maze showed a significant ($p < 0.025$) cannabis smoke extract effect in female offspring during sessions 1–6 (Fig. 3). Post-hoc Dunnett’s tests showed that the female offspring of the late CSE-exposed males (12.8 ± 0.6) had significantly ($p < 0.01$) more errors than control females (10.7 ± 0.5). The early exposure group (11.6 ± 0.3) did not significantly differ from controls.

For reference memory errors, there was an interaction of paternal cannabis smoke extract x sex ($F(2,24) = 3.37, p < 0.06$) that prompted tests of the simple main effects of cannabis smoke extract exposure in males and females, however, no significant paternal cannabis smoke extract effects were seen either the males or females.

Response latency in the radial-arm maze was also assessed. There was a paternal cannabis smoke extract x sex interaction ($F(2,24) = 2.91, p < 0.08$) that called for tests of paternal cannabis smoke extract within each sex. However, these follow-up tests for paternal cannabis smoke extract for males and females did not detect significant effects in either sex.

3.7. Operant visual signal detection test of learning and attention

There was a significant interaction of paternal cannabis smoke extract x error type interaction ($F(2,24) = 8.16, p < 0.005$). Controls had $82.7 \pm 1.5\%$ correct hit and $88.2 \pm 1.6\%$ correct rejection, the early paternal cannabis smoke extract group had $85.10 \pm 1.3\%$ correct hit and $86.1 \pm 1.4\%$ correct rejection and the late paternal cannabis smoke extract offspring had $82.8 \pm 1.6\%$ correct hit and $91.9 \pm 1.1\%$ correct rejection. Follow-up Dunnett’s tests within the simple main effects of paternal cannabis smoke extract exposure on percent correct with percent hits and percent correct rejections did not detect any significant differences between the treated and control groups. There were also no significant differences seen in the number of omissions or “no response” trials.

4. Discussion

Paternal cannabis smoke extract exposure produced long-lasting behavioral changes in the offspring of the exposed male rats. Significant behavioral effects relative to control were seen with both groups with paternal cannabis smoke extract exposure, ending either three or 56 days before mating. Paternal rats with “late exposure” were exposed to cannabis smoke extract for 28 days during the later stages of sperm maturation, matching the time period we previously investigated with THC alone. Paternal rats with “early exposure” were exposed to cannabis smoke extract for 28 days, followed by an eight-week period of drug abstinence between the end of chronic cannabis smoke extract exposure and conception. We hypothesized that the early exposure group would experience a full sperm cycle recovery leading to less behavioral deficits. Behavioral analyses of the offspring indicated that while CSE exposure during these two periods of sperm development each led to heritable behavioral effects, the exact nature of those effects were somewhat distinct.

The clinical health of the offspring was not significantly affected by paternal exposure during either time period; however, these rats did show alterations in anxiolytic behavior, spatial learning and working memory when compared to controls. Specifically, on the elevated plus

**Paternal Cannabis Exposure Effects on Offspring Behavior
16-Arm Radial Maze: Working Memory Errors**

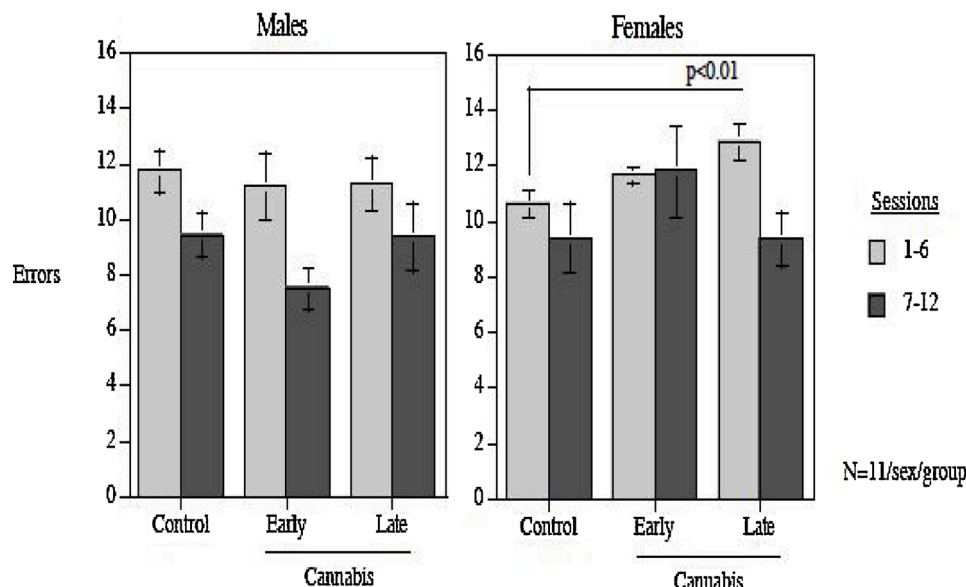


Fig. 3. 16-arm radial maze working memory errors (mean ± sem).

maze, the offspring of males with either early or late cannabis smoke extract exposure spent significantly more time on the open arms than control offspring, indicative of greater risk-taking behavior. This interpretation reflects the risk of predation that rats face in their natural environments, and the way that thigmotaxis, or preferences for closed spaces, reduces this risk. These results share similarities with that of young adult cannabis users, who have reported greater risk-taking behavior in social, health and safety, and ethical domains (Gilman et al., 2015). Additionally, higher levels of cannabis or THC use have also been associated with a decrease in threat-related amygdala reactivity which may explain some of the anxiolytic effects seen in the current study. (Cornelius et al., 2010; Phan et al., 2008).

There were also significant differences seen in the novel-object recognition test of non-spatial memory. The session was divided into two 5-min time blocks to allow patterns of within session learning to be assessed, measured as a change in novel object investigation as the object became more familiar. Male offspring of the late cannabis smoke extract exposure group had a significantly greater drop-off of interest in the novel object than controls. By contrast, males in the early cannabis smoke extract exposure group did not have a significant effect on this measure. A faster rate of investigation loss indicates an underlying difference in the learning processes that update the salience of objects over time. No significant effects were seen in female offspring. Increased variability of response was seen in females which may be due to the changes in novel-object recognition performance across the estrus cycle (Walf et al., 2006). As observed in the present study, a reduced ability to maintain attention on a preferred object shares some similarities to problems experienced by those with inattentive-type ADHD (Song and Hakoda, 2014).

Another cognitive test affected by paternal cannabis smoke extract exposure was the 16-arm radial maze. This test of spatial learning and memory revealed sex-specific effects of paternal cannabis smoke extract exposure on working memory errors during sessions 1–6 (Fig. 3). Although the early exposure group (56-day interval) did not differ from controls, female offspring of the late exposure group had significantly more working memory errors than control females. It is important to note that in our previous study where male rats underwent a 28-day chronic subcutaneous THC exposure prior to mating, rather than CSE, offspring in the exposure group also showed a significant drop-off in interest of the novel object during the NOR test, along with a significant delay in learning during the radial arm test (Holloway et al., 2020). In both studies, spatial long-term memory was spared, suggesting that paternal cannabis smoke extract exposure may be more associated with changes in executive functions like working memory than with other forms of learning and memory.

Within the two previous mentioned cognitive tasks, sex differences in response were prominent. The present study suggests that differences in response may extend to sperm well before sex is determined. A combination of genetic and developmental factors may play roles in these sex differences. It is not yet known which heritable epigenetic changes in sperm are on sex-determining genes, or play a role in sexually dimorphic processes, although future mechanistic work on this question could be quite informative. With respect to development, it is known that many systems of the brain show a degree of sexual dimorphism, including those that mediate the response to cannabis. For example, female rats have been found to be more sensitive than males to THC-induced impairment of memory as well as sensitivity to pain, motor activity, and reinforcing efficacy (Cha et al., 2007; Craft et al., 2013). As these differences unfold during development, epigenetic changes before conception may have unique effects. Since our early exposure group was not found to be significantly different from controls on each of these outcomes, these differential effects appear to be most associated with changes that persist within mature sperm. Our 56-day interval cannabis smoke extract exposure group may have had sufficient time to recover from sperm modifications that affect memory of offspring, whereas the group with 28-day CSE exposure ending shortly before conception did not

have time for these biological readjustments. Thus, paternal cannabis smoke extract exposure during the early proliferative stages of sperm maturation could exert less deleterious effects on neurodevelopment of the offspring than exposure during the late maturation phases of sperm development.

Sample sizes of $N = 11$ litters per paternal treatment condition were sufficient to detect significant long-term behavioral dysfunction in the offspring. Larger sample sizes may have provided increased statistical power for detecting more subtle behavioral impacts from paternal exposure.

Behavioral dysfunctions seen with rats from in the current study could arise from developmental disruption to the cholinergic system, which exhibits bidirectional crosstalk with the endocannabinoid system (Scherma et al., 2016). We have previously found that paternal THC exposure can cause significant alterations in the developmental trajectory of acetylcholine systems in the offspring, which provide essential inputs for reward, cognition, and mood (Slotkin et al., 2020). Additionally, the effects of cannabis are mediated by cannabinoid receptors, and paternal exposure has the potential to epigenetically affect many of the same neural processes including addiction, reward, motor skills, cognition and stress through alterations to the endocannabinoid system (Ferland and Hurd, 2020). Following this, receptors which mediate the actions of THC are predominately expressed in the mesocorticolimbic system in the prenatal brain (Hurd et al., 2019). The mesocorticolimbic system is a part of the brain's motivational circuit, in which the medial network of the frontal cortex projects not only to the nucleus accumbens, but also the striatum which is highly interconnected with the amygdala (Ikemoto et al., 2015). Studies in rats and prenatal humans found that these systems may experience disruption after intergenerational exposure to marijuana and THC, providing a foundation for alterations seen in the current study concerning anxiolytic behavior, spatial learning and working memory when compared to controls (Szutorisz et al., 2016; Wang et al., 2004; Watson et al., 2015).

These findings emphasize the urgent need for more research dedicated to investigating risks associated with paternal exposure to cannabis prior to conception and the role of the endocannabinoid system during gestation to determine if there are long-term consequences to offspring health and neurodevelopment. However, results of the current study should be considered along with several important limitations. First, it is presently unclear whether DNA methylation changes produced from environmental pressures, such as social behavior or stress, were passed on to the offspring through the maternal or paternal lineage. Recent studies have shown that paternal exposure to stress can dysregulate DNA methylation, alter the small noncoding RNA profile in sperm, and modify depressive and anxiety phenotypes in the offspring (Rodgers et al., 2013; Short et al., 2016). Further, it has been demonstrated that chronic exposure to cannabinoids in male rats during adolescence can lead to epigenetic reprogramming which may increase the vulnerability to stress-induced anxiety in the offspring (Andaloussi et al., 2019). Therefore, it is possible that the males injected with CSE exhibited some form of social change away from the norm when interacting with females during mating, spurring epigenetic modifications in the genome leading to some of the behavioral changes seen in the current study.

Additionally, in our previous work we examined the effects of 4 mg/kg pure THC on identical behavioral tests and saw differing results when comparing 4 mg/kg THC in CSE. This could be attributed to the composition of CSE, which contains cannabidiol, cannibol, and thousands of other unknown compounds. It is difficult to decipher which type of epigenetic modifications to the genome these toxins could produce in combination and if they could lead to variable behavioral phenotypes. Through the maternal line, cannabinoid receptor activation has been shown to induce transgenerational effects even in the absence of in utero exposure. Male offspring of female rats who were treated in adolescence with cannabinoid agonists exhibited enhanced morphine-induced conditioned place preference and locomotor sensitization

(Byrnes et al., 2012). Cannabinoid exposure via the paternal line through treatment of CSE is likely to produce peculiar behavioral outcomes as well. Parental germline THC exposure alone has been shown to cause changes in DNA methylation within the nucleus accumbens of the offspring, and others have suggested alterations in behavior arise from changes in the mRNA expression of cannabinoid, dopamine and glutamatergic receptor genes in the striatum (Pitsilis et al., 2017; Szutorisz et al., 2014; Watson et al., 2015). Thus, the contents of cannabis smoke extract used in the current study have the potential to disrupt a myriad of neural processes leading to behavioral change.

Lastly, because behavioral tests were performed sequentially on the same offspring, there is the potential confound of age at the time of the test. Our previous research has shown that when tested as adults, rats may produce different results than when they were tested in adolescence (Holloway et al., 2020). For example, offspring of males exposed to 2 mg/kg/day of THC exhibited significant locomotor hyperactivity during adolescence, but this effect was no longer evident when tested again in adulthood. Thus, based on the age of the offspring at the time of the various tests in the current study, some effects may have been missed due to age or experience-induced recovery.

Overall, these data and others contribute evidence that paternal exposure to cannabis smoke extract before conception can produce behavioral alterations in the offspring that persist into adulthood. Additionally, alterations in the timing of exposure within the spermatogenic cycle can produce different effects in memory and anxiety systems of the progeny. Future studies should facilitate a comparison of cannabis smoke extract and pure THC to determine if each of these compounds produces similar sperm DNA methylation changes to better understand disruptions in the behavior of the offspring.

CRedit author statement

Zade 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.

Alexandra K. Torres tested the subjects and helped write the article.

Janequia Evans tested the subjects and helped write the article.

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

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

Vaishnavi Katragadda tested the subjects and helped write the article.

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

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

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

Amir H. Rezvani 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

The authors declare no conflict of interest.

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