Repeated ∆9-tetrahydrocannabinol (THC) vapor inhalation during adolescence: Sex differences in acute thermoregulatory tolerance and in feeding during adulthood.

Jacques D. Nguyen, K. M. Creehan, Tony M. Kerr, Michael A. Taffe

Department of Neuroscience; The Scripps Research Institute; La Jolla, CA, USA

Running Head: Repeated adolescent THC inhalation

Address Correspondence to: Dr. Michael A. Taffe, Department of Neuroscience, SP30-2400; 10550 North Torrey Pines Road; The Scripps Research Institute, La Jolla, CA 92037; USA; Phone: +1.858.784.7228; Fax: +1.858.784.7405; Email: mtaffe@scripps.edu

Abstract:

Many US adolescents are regularly exposed to Δ^9-tetrahydrocannabinol (THC) via the smoking and more recently, vaping, of cannabis and cannabis extracts. Growing legalization of cannabis for medical and recreational purposes, and decreasing perceptions of harm, make it increasingly important to determine the consequences of frequent adolescent exposure.

Male and female Wistar rats were implanted with radio-telemetry devices to monitor body temperature and exposed to twice daily 30 minute inhalation of THC vapor, or the propylene glycol (PG) vehicle, from post-natal day (PND) 35-39 and PND 42-45 using an e-cigarette based system. Responses to THC were re-determined from PND 86-94 and chow intake was assessed in mid adulthood. Blood samples were obtained from additional groups following THC inhalation on PND 31, on PND 39 after a week of twice daily exposure, and during early adulthood. Additional groups of male rats exposed repeatedly to THC or PG during adolescence were evaluated for intravenous self-administration of oxycodone as adults.

Female, but not male, adolescents developed tolerance to the hypothermic effects of THC inhalation in the first week of repeated exposure despite similar plasma THC levels. Each sex exhibited tolerance to THC hypothermia in adulthood after repeated adolescent THC with THC exhibiting greater potency in females. Repeated-THC male rats consumed more food than their PG treated control group, in the absence of a significant bodyweight difference, but male rats did not differ in oxycodone self-administration.

This study confirms that repeated THC vapor inhalation in adolescence results in lasting consequences observable in adulthood.

Keywords: temperature, cannabinoid, e-cigarette, intrapulmonary, vape

Introduction

Significant numbers of US adolescents are exposed to Δ^9-tetrahydrocannabinol (THC) on a regular basis via the smoking and, more recently vaping, of cannabis and/or cannabis extracts. Data from the Monitoring the Future survey confirm that 5-6% of 12th grade students use cannabis nearly daily and 13.9% have used in the past month (Miech *et al*, 2018). About 10% of 12th grade students have *vaped* cannabis at least once in the past year and 5% in the past month (*ibid*). Furthermore, growing legalization of cannabis use for medical and recreational purposes, and a decreasing perception of harm, suggests these populations will only grow in coming years. It is therefore of significant interest to determine the consequences of frequent adolescent exposure to THC.

Repeated treatment of adolescent rats with THC by injection results in observable lasting effects in adulthood including decreased bodyweight (Rubino *et al*, 2008), impaired spatial working memory (Rubino *et al*, 2009), increased heroin self-administration (Ellgren *et al*, 2007), increased reinstatement of heroin seeking after a pharmacological stressor (Stopponi *et al*, 2014) and greater sensitivity to learning impairment produced by acute THC (Winsauer *et al*, 2011). This shows the potential risks of repeated THC exposure, however, prior investigations have primarily employed the injected route of administration (Cha *et al*, 2007; Ellgren *et al*, 2007; Rubino *et al*, 2008; Winsauer *et al*, 2011) which may not match the human condition very well. For example, the duration of effect of THC on, e.g., hypothermia, in the rat lasts hours longer following i.p. administration compared to a vapor inhalation regimen that produces a similar temperature nadir and peak plasma THC levels (Nguyen *et al*, 2016a; Taffe *et al*, 2015). Thus, the route of administration of THC may be critical to the overall outcome. The majority of human use of cannabis is via inhalation which entails, comparatively, a more rapid onset and offset with a shorter overall duration of activity. Thus, the study of inhaled delivery of cannabis constituents in rodent models may support improved translational inferences.

The goal of this study was first to determine if vapor inhalation of THC reduces body temperature during adolescence in the rat. The e-cigarette based model has been validated previously only in adult rats (Javadi-Paydar *et al*, 2018; Nguyen *et al*, 2016a) and therefore basic efficacy in adolescents needed to be established. The second, and major, goal was to determine if twice daily THC over consecutive days in adolescence produces tolerance, which is a key initial indicator of THC exposure sufficient to induce lasting

changes in the central nervous system. Our recent study in adult rats showed tolerance to the hypothermic and antinociceptive responses to THC after twice (female) or thrice (male or female) daily inhalation (Nguyen *et al*, 2018).The third goal was to test the hypothesis that the development of tolerance differs across rat sex, since prior work has shown that female rats develop tolerance more rapidly and at a lower mg/kg adjusted dose following twice-daily parenteral injection of relatively high THC doses (Wakley *et al*, 2014). Our prior work shows that male and female adult rats achieve similar plasma THC levels after identical inhalation conditions (Javadi-Paydar *et al*, 2018) but that female rats become tolerant with less intensive THC exposure compared with males (Nguyen *et al*, 2018). Thus it was hypothesized that the female adolescent rats would be more sensitive than the males, developing tolerance more rapidly and more completely. A final goal was to determine if there were lasting consequences of repeated adolescent THC inhalation in the adult rat in terms of tolerance to acute THC exposure, alterations in weight gain and feeding behavior (Sofia and Barry, 1974) and the propensity to self-administer an opioid drug, oxycodone, as has previously been reported for heroin (Ellgren *et al*, 2007; Stopponi *et al*, 2014).

Methods

Subjects: Female (N=24) and male (N=48) Wistar rats (Charles River) were shipped from the vendor on postnatal day (PND) 19 and entered the laboratory at PND 22. Rats were housed in humidity and temperature-controlled (23±2 °C) vivaria on 12:12 hour light:dark cycles and all studies were conducted in the rats' scotophase. Animals had *ad libitum* access to food and water in their home cages. Gelatin nutritional support (DietGel® Recovery, ClearH2O, Westbrook, ME, USA) was provided weekdays PND 32-46. All procedures were conducted under protocols approved by the Institutional Care and Use Committee of The Scripps Research Institute.

***Radio-telemetry*:** Rats (N=16 per sex) were implanted with sterile radio-telemetry transmitters (Data Sciences International, St Paul, MN; TA11TA-F20) in the abdominal cavity as previously described (Taffe *et al*, 2015; Wright *et al*, 2012) on PND 25. Animals were recorded in either the vapor inhalation chambers (male rats) or in separate clean home cages (female rats) in a dark testing room, separate from the vivarium. This difference in

procedure means that a 30 minute recording time point was available for male rats but not for female rats. Radiotelemetry transmissions were collected via telemetry receiver plates (Data Sciences International, St Paul, MN; RPC-1 or RMC-1) placed under the cages as described in prior investigations (Aarde *et al*, 2013; Miller *et al*, 2013; Wright *et al*, 2012). The order and details of studies are outlined in **Table 1**.

Drugs: Δ^9-tetrahydrocannabinol (THC) was administered by vapor inhalation with doses described by the concentration in the propylene glycol (PG) vehicle, puff schedule and duration of inhalation sessions. The THC was provided by the U.S. National Institute on Drug Abuse and the PG was obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA).

Inhalation procedure: The inhalation procedure followed methods that have been recently described (Javadi-Paydar *et al*, 2018; Nguyen *et al*, 2016a). Sealed exposure chambers were modified from the 259mm X 234mm X 209mm Allentown, Inc (Allentown, NJ) rat cage to regulate airflow and the delivery of vaporized drug to rats. An e-vape controller (Model SSV-1; La Jolla Alcohol Research, Inc, La Jolla, CA, USA) was triggered to deliver the scheduled series of puffs from Protank 3 Atomizer (Kanger Tech; Shenzhen Kanger Technology Co.,LTD; Fuyong Town, Shenzhen, China) e-cigarette cartridges by a computerized controller designed by the equipment manufacturer (Control Cube 1; La Jolla Alcohol Research, Inc, La Jolla, CA, USA). Type 2 sealed exposure chambers (La Jolla Alcohol Research, Inc; La Jolla, CA, USA) and second generation e-vape controllers (Model SSV-2; La Jolla Alcohol Research, Inc, La Jolla, CA, USA) with Herakles Sub Ohm Tank e-cigarette cartridges (Sense; Shenzhen Sense Technology Co., LTD; Baoan Dist, Shenzhen, Guangdong, China) by MedPC IV software (Med Associates, St. Albans, VT USA) were used for THC exposure in the groups destined for the pharmacokinetic and oxycodone self-administration experiments. The chamber air was vacuum-controlled by a chamber exhaust valve (i.e., a “pull” system) to flow room ambient air through an intake valve at ~1 L per minute. This also functioned to ensure that vapor entered the chamber on each device triggering event. The vapor stream was integrated with the ambient air stream once triggered. For all studies the system delivered four 10-s vapor puffs, with 2-s intervals, every 5 minutes for 30 minutes.

Feeding procedure: To begin a test day, all chow was removed at the start of the dark cycle in the vivarium. Rats were moved to a procedure room, weighed and placed individually in separate rat cages equipped with J-style hanging chow dispensers (Guinea Pig Feeder; Ancare, Bellmore, NY, USA) starting 2 h into the dark period. Chow was removed and weighed every 2 hours for 6 hours; rats were weighed again at the end of the test interval.

Table 1. Order of inhalation and feeding studies for male and female radio-telemetry cohorts.

Female and Male	PND 29	Air inhalation for 30 minutes
	PND 30	PG vapor inhalation for 30 minutes
	PND 31	THC (100 mg/mL) vapor inhalation for 30 minutes
	PND 35-39, 42-45	PG or THC (100 mg/mL) vapor inhalation for 30 minutes, b.i.d., qa. 5 h
	PND 85	PG vapor inhalation for 30 minutes
	PND 86	THC (100 mg/mL) vapor inhalation for 30 minutes
Female only	PND 91-94	THC (25, 50 mg/mL) vapor inhalation
	PND 105-109	*feeding study*
	PND 168-179	*feeding study*
Male only	PND 93-94	PG and THC (200 mg/mL) vapor inhalation in balanced order
	PND 100-101	PG and THC (200 mg/mL) vapor inhalation for 40 minutes in balanced order
	PND154-165	*feeding study*

***Pharmacokinetics*:** Separate groups (N=8) of male and female rats were received on PND 22 and received a single inhalation session (THC 100 mg/mL; 30 minutes) on PND 31, after which a blood sample (~500 uL) was obtained by acute venipuncture under inhalation anesthesia. The rats then received twice daily inhalation sessions from PND 36-39, as in the above experiment, with a second blood sample obtained after the first session on PND 39. This timing was designed to capture the first session of complete tolerance observed in the female rats in the telemetry study. Blood samples were also obtained from these groups on PND 86 following a THC 100 mg/mL (30 minutes) inhalation session and on PND 100 and 107 following THC 50 or 200 mg/mL inhalation for 30 minutes in a counterbalanced order.

Blood samples were collected (~500 ul) via jugular needle insertion under anesthesia with an isoflurane/oxygen vapor mixture (isoflurane 5% induction, 1–3% maintenance) 35 minutes post-initiation of vapor inhalation. Plasma THC content was quantified using fast liquid chromatography/mass spectrometry (LC/MS) adapted from (Irimia *et al*, 2015; Lacroix and Saussereau, 2012; Nguyen *et al*, 2018). 5 µL of plasma

were mixed with 50 µL of deuterated internal standard (100 ng/mL CBD-d3 and THC-d3; Cerilliant), and cannabinoids were extracted into 300 µL acetonitrile and 600 µL of chloroform and then dried. Samples were reconstituted in 100 µL of an acetonitrile/methanol/water (2:1:1) mixture. Separation was performed on an Agilent LC1100 using an Eclipse XDB-C18 column (3.5um, 2.1mm x 100mm) using gradient elution with water and methanol, both with 0.2 % formic acid (300 µL/min; 73-90%). Cannabinoids were quantified using an Agilent MSD6140 single quadrupole using electrospray ionization and selected ion monitoring [CBD (m/z=315.2), CBD-d3 (m/z=318.3), THC (m/z=315.2) and THC-d3 (m/z=318.3)]. Calibration curves were conducted for each assay at a concentration range of 0-200 ng/mL and observed correlation coefficients were 0.999.

Adolescent Vapor Exposure. The adolescent rats were divided into two groups of 12 which received 30 minute episodes of vapor exposure, b.i.d., qa. 5 h, in quads to either THC (200 mg/mL) or the propylene glycol (PG) vehicle on sequential days PND 35-39 and again on PND 42-46. These groups received intravenous catheter implant surgery on PND 84-87 and initiated IVSA of oxycodone (0.15 mg/kg/infusion; 8 h sessions; Fixed Ratio 1 response contingency) on PND 112. Following an acquisition interval of 17 sessions the rats completed six sessions of Fixed Ratio (8 h) and Progressive Ratio (3 h) dose substitution (0.006, 0.06, 0.15 mg/kg/infusion) in a counter-balanced order.

Intravenous Catheterization. Rats were anesthetized with an isoflurane/oxygen vapor mixture (isoflurane 5% induction, 1-3% maintenance) and prepared with chronic indwelling intravenous catheters as described previously (Aarde *et al*, 2017; Miller *et al*, 2015; Nguyen *et al*, 2016b). Briefly, the intravenous catheters consisted of a 14.5-cm length of polyurethane based tubing (Micro-Renathane®, Braintree Scientific, Inc, Braintree, MA) fitted to a guide cannula (Plastics One, Roanoke, VA) curved at an angle and encased in dental cement anchored to an ~3 cm circle of durable mesh. Catheter tubing was passed subcutaneously from the animal's back to the right jugular vein. Catheter tubing was inserted into the vein and tied gently with suture thread. A liquid tissue adhesive was used to close the incisions (3M™ Vetbond™ Tissue Adhesive: 1469SB, 3M, St. Paul, MN). A minimum of 4 days was allowed for surgical recovery prior to starting an experiment. For the first three days of the recovery period, an antibiotic (cefazolin) and an analgesic (flunixin) were

administered daily. During testing and training, intravenous catheters were flushed with ~0.2-0.3 ml heparinized (166.7 USP/ml) saline before sessions and ~0.2-0.3 ml heparinized saline containing cefazolin (100 mg/mL) after sessions. Catheter patency was assessed once a week after the last session of the week, via administration through the catheter of ~0.2 ml (10 mg/ml) of the ultra-short-acting barbiturate anesthetic Brevital sodium (1% methohexital sodium; Eli Lilly, Indianapolis, IN). Animals with patent catheters exhibit prominent signs of anesthesia (pronounced loss of muscle tone) within 3 sec after infusion. Animals that failed to display these signs were considered to have faulty catheters, and if catheter patency failure was detected, data that were collected after the previous passing of this test were excluded from analysis.

***Oxycodone Self-Administration*:** Intravenous self-administration was conducted in operant boxes (Med Associates) located inside sound-attenuating chambers located in an experimental room (ambient temperature 22 ± 1 °C; illuminated by red light) outside of the housing vivarium. To begin a session, the catheter fittings on the animals' backs were connected to polyethylene tubing contained inside a protective spring suspended into the operant chamber from a liquid swivel attached to a balance arm. Each operant session started with the extension of two retractable levers into the chamber. Following each completion of the response requirement (response ratio), a white stimulus light (located above the reinforced lever) signaled delivery of the reinforcer and remained on during a 20-sec post-infusion timeout, during which responses were recorded but had no scheduled consequences. Drug infusions were delivered via syringe pump. The training dose (0.15 mg/kg/infusion; ~0.1 ml/infusion) was selected from prior oxycodone self-administration studies (Nguyen et al, 2018; Wade et al, 2015). The rats (N=12 per group) were trained in 8 h sessions using a Fixed Ratio 1 response contingency during weekdays (5 days per week).

In the PR paradigm, the required response ratio was increased after each reinforcer delivery within a session (Hodos, 1961; Segal and Mandell, 1974) as determined by the following equation (rounded to the nearest integer): Response Ratio=5e^(injection number*j)–5 (Richardson and Roberts, 1996). The j value was set to 0.2.

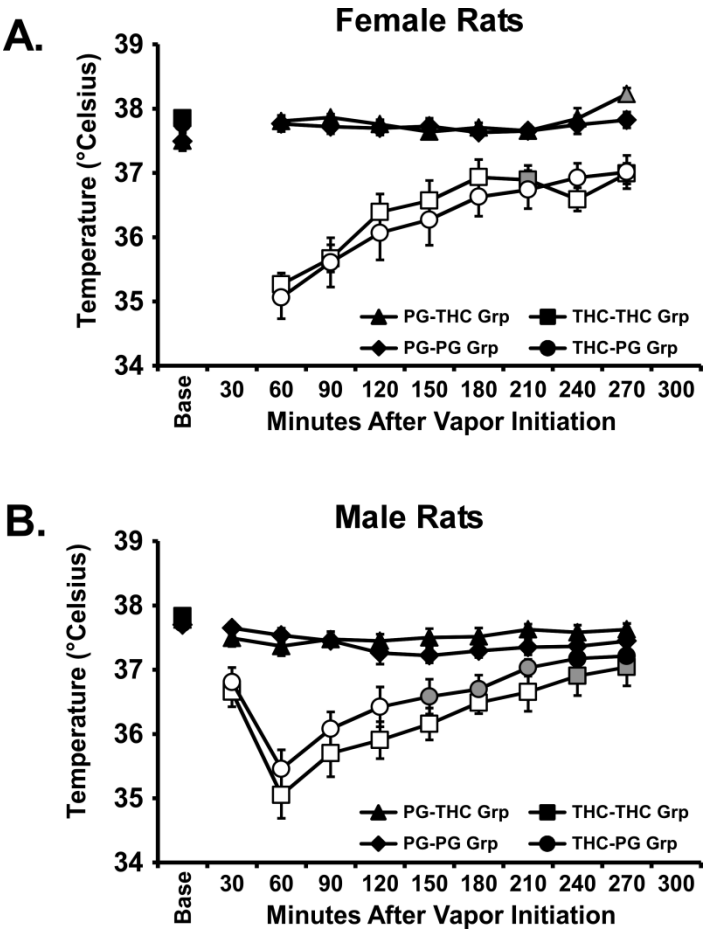

Figure 1: *Mean (N=8 per group; ±SEM) body temperature responses after inhalation of PG or THC (100mg/mL) vapor for 30 minutes in the eventual repeated-PG or repeated-THC A) female and B) male groups. Open symbols indicate a significant difference from both vehicle at a given time-point and the within-treatment baseline, while shaded symbols indicate a significant difference from the baseline only.*

Data Analysis: The body temperature and activity rate (counts per minute) were collected on a 5-minute schedule but are expressed as 30 min averages for analysis in the study. The time courses for data collection are expressed relative to the start of the inhalation. Any missing temperature values were interpolated from the values before and after the lost time point, this typically involves fewer than 10% of observations. Missing activity rates were not replaced because 5-min to 5-min values can change dramatically, thus there is no justification for interpolating. Statistical analysis of temperature, activity, plasma THC concentrations, bodyweight and infusions earned (IVSA) was conducted with Analysis of Variance (ANOVA). Between-groups factors included Sex or Adolescent treatment condition, as appropriate. Within-subjects factors of Session, Post-natal day (PND) and Vapor inhalation condition were included. Data were analyzed with Analysis of Variance (ANOVA) including repeated measures factors for the treatment condition and the time post-injection and between-subjects factors for Group. Any significant main effects were followed with post hoc analysis using Tukey (multi-level factors) or Sidak (two-level factors) correction for multiple comparisons. All analysis used Prism 6 or 7 for Windows (v. 6.02, 7.03; GraphPad Software, Inc, San Diego CA).

Results:

THC-induced hypothermia in adolescent rats

The initial experiment confirmed that vapor inhalation of THC induces hypothermia in adolescent rats of each sex (**Figure 1A, B**). Furthermore, the male and female groups destined for repeated-THC versus repeated-PG were identical in response to PG on PND 30 and to THC on PND 31. The three-factor analysis confirmed significant effects of Vapor inhalation condition [$F(1,252)=310.1$; $p<0.0001$], of Time after vapor initiation [$F(8,252)=12.58$; $p<0.0001$] and the interaction of Time with Vapor inhalation condition [$F(8, 252)=16.13$; $p<0.0001$] in female rats; there were no significant main effects or interactions with the eventual repeated-exposure groups. The three-way analysis for the male groups confirmed significant effects of Vapor inhalation condition [$F(1,280)=215.1$; $p<0.0001$], of Time after vapor initiation [$F(9,280)=16.19$; $p<0.0001$] and the interaction of Time with Vapor inhalation condition [$F(9,280)=10.61$; $p<0.0001$] and the interaction of eventual repeated-exposure Group with Vapor inhalation condition [$F(1,280)=10.22$; $p=0.0015$]. Post hoc analysis of this latter interaction failed to confirm any differences between eventual repeated-PG and repeated-THC groups in either PG or THC inhalation conditions.

Follow up analysis of the temperature 60 minutes after the start of inhalation including the repeated-PG and repeated-THC cohorts of each sex confirmed a main effect of vapor inhalation condition [$F(1,28)=249.1$; $p<0.0001$] but not of group or of the interaction. The post hoc test confirmed a significantly lower temperature after THC vapor compared with PG within each group, and did not confirm any differences across all the groups within PG or THC vapor inhalation conditions.

Effect of repeated THC or PG inhalation on hypothermia in adolescent rats

There were no changes in the body temperature of the repeated-PG groups across the recording interval for any of the recording days (**Supplemental Figure S1**). The male and female rats in the repeated-THC groups became hypothermic following inhalation during each of the chronic exposure weeks (**Figure 2**). Tolerance to the hypothermic effects differed between the sexes. Statistical analysis of the female animal's temperature confirmed significant effects of Time after vapor initiation and Day in Week 1 [Time:

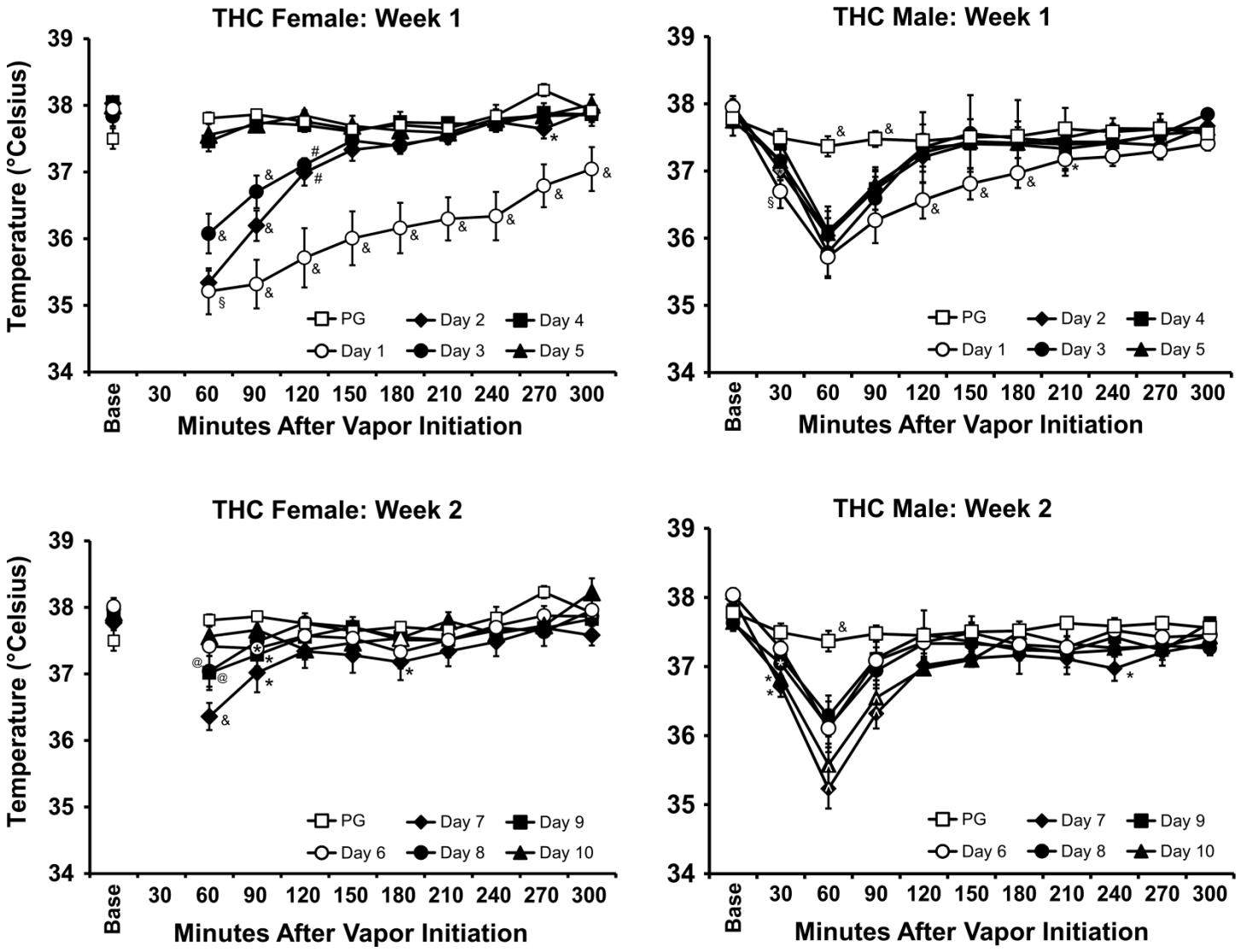

Figure 2: *Mean (N=8 per sex; ±SEM) body temperature recorded for the first vapor inhalation session of each day is depicted for the repeated THC groups. The PND 30 PG data are depicted in upper and lower panels. A significant difference from all other days at a given time after the start of inhalation is depicted by &, a significant difference from all days except Day 2 by §, a significant difference from PG, Day 4 and 5 by #, a significant difference from PG, Day 7 and 10 by @, a significant difference from PG, Days 6, 8 and 9 by ^, and a significant difference from PG by *.*

F(9,63)=32.61; *p*<0.0001; Day: F(5,35)=18.72; *p*<0.0001; Interaction: F(45,315)=11.39; *p*<0.0001] and Week 2 [Time: F(9,63)=9.69; *p*<0.0001; Day: n.s.; Interaction: F(45,315)=3.12; *p*<0.0001]. Statistical analysis of the male animal's temperature confirmed significant effects in Week 1 [Time: F(10,70)=31.26; *p*<0.0001; Day: F(5,35)=4.35; *p*<0.005; Interaction: F(50,350)=4.72; *p*<0.0001] and Week 2 [Time: F(10,70)=31.62; *p*<0.0001; Day: F(5,35)=6.467; *p*<0.0005; Interaction: F(50,350)=4.28; *p*<0.0001]. The post hoc test confirmed that a progressive tolerance to hypothermia developed in the female rats across the first 4 days of exposure with no hypothermia observed on days 4 and 5. The post hoc test also confirmed that significant tolerance developed

in Days 3-5 relative to Day 1 in the males, although this was limited to the 120-180 minute time points. In Week 2, the post hoc test confirmed a significant reduction in body temperature relative to the PG day 60 minutes after the start of inhalation on Days 7-9 for the female rats and Days 6-10 for the male rats. Follow up analysis was conducted on the temperature recorded 60 minutes after the start of inhalation for repeated-THC cohorts of each sex to directly compare the magnitude of hypothermia across days and sex (**Figure 3**). The ANOVA confirmed a main effect of day [$F(11,154)=19.14$; $p<0.0001$] of Sex [$F(1,14)=10.6$; $p<0.01$] and of the interaction [$F(11,154)=6.36$; $p<0.0001$]. The post hoc test further confirmed that significant reductions in temperature following THC inhalation (relative to the PG condition) were observed for each THC session in male rats. Significant reductions in body temperature were also confirmed for female rats on PND days 31, 35-37 and 43 and a significant sex difference were confirmed for PND 38-42 and 46. A reduction in body temperature was observed during the first daily session of all 10 days of repeated THC inhalation but no change in body temperature was observed during PG inhalation.

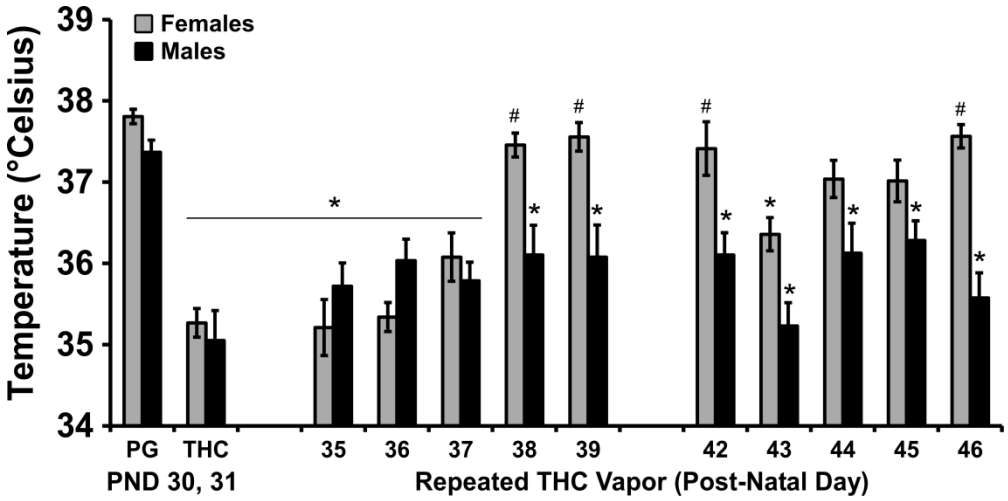

Figure 3: *Mean (N=8 per sex; ±SEM) body temperature 60 minutes after the start of inhalation for all inhalation days during adolescence. A significant difference from the PG value, within group, is indicated with * and a significant sex difference by #.*

Adult THC inhalation

Tolerance to the hypothermic effects of THC inhalation persisted into adulthood in the repeated-THC groups (**Figure 4**). The statistical analysis of the temperature of the repeated PG females after inhalation during adulthood confirmed a significant effect of Time after vapor initiation [$F(8,56)=13.63$; $p<0.0001$], of Vapor Dose condition [$F(3,21)=20.87$; $p<0.0001$] and of the interaction of Time with Vapor Dose condition [$F(24,168)=2.85$; $p<0.0001$]. Analysis of the temperature of the repeated THC females confirmed a significant effect of Time

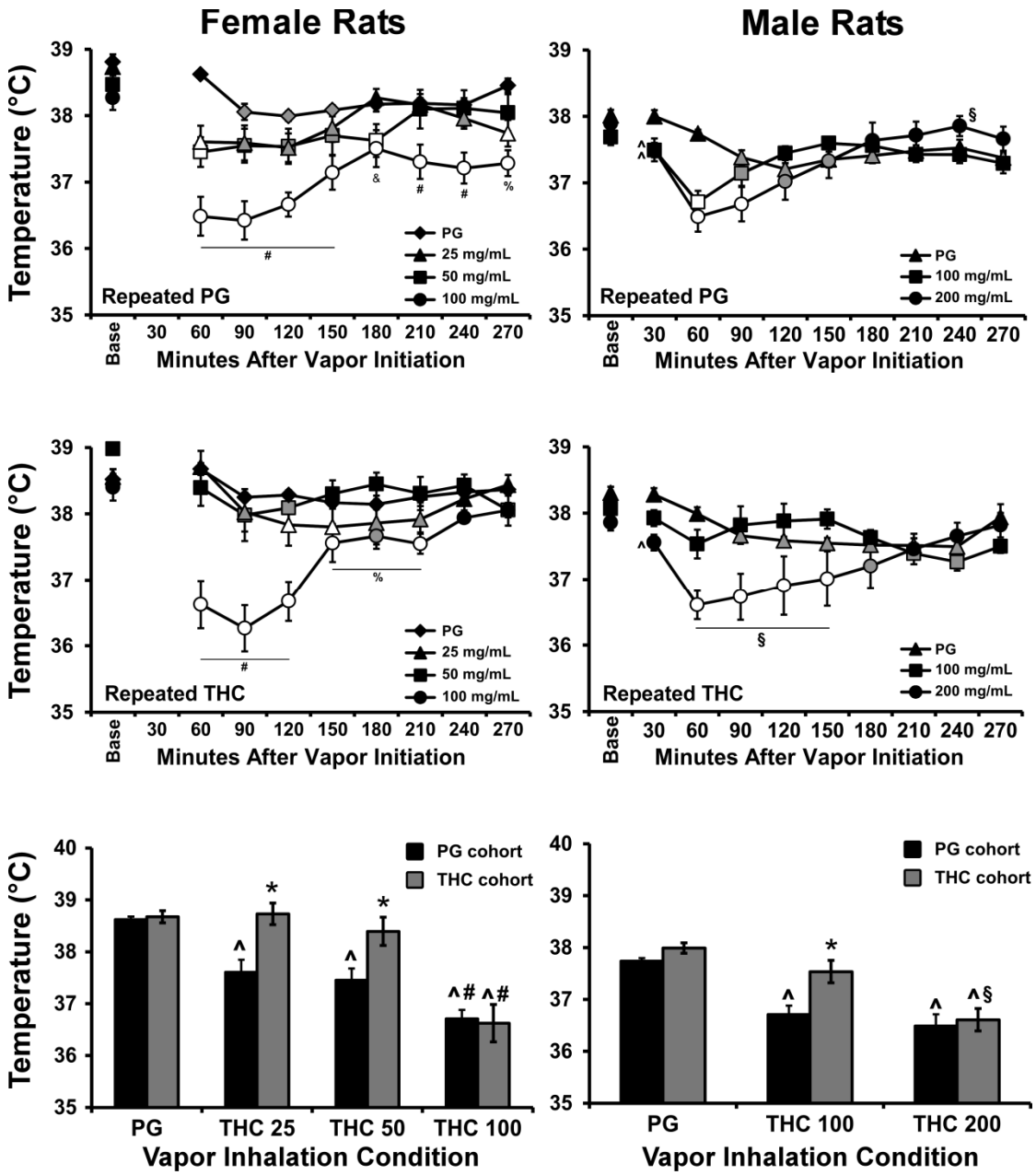

***Figure 4**: Mean (SEM) body temperature of female and male rat cohorts (N=8 per group) exposed to repeated PG or THC during adolescence and challenged in acute sessions with vapor from PG or varying concentrations of THC (25-200 mg/mL) from PND 85-94. Open symbols indicate a significant difference from the pre-inhalation baseline (Base) and the corresponding time after PG inhalation and grey symbols represent a difference from the baseline only. A significant difference from the PG condition is indicated with ^, from the 25 and 50 mg/mL condition with #, from the 100 mg/mL condition by §, from the 50 mg/mL condition by % and from the 25 mg/mL condition by &. A significant difference between treatment groups, within sex, is indicated with *.*

after vapor initiation [F(8,56)=8.05; $p<0.0001$], of Vapor Dose condition [F(3,21)=13.93; $p<0.0001$] and of the interaction of factors [F(24,168)=6.29; $p<0.0001$]. The analysis of the temperature of the repeated PG males confirmed a significant effect of Time after vapor initiation [F(9,63)=18.24; $p<0.0001$] and of the interaction of

Time with Vapor Dose condition [F(18,126)=6.75; p<0.0001]. Analysis of the temperature of the repeated THC males confirmed a significant effect of Time after vapor initiation [F(9,63)=11.35; p<0.0001], of Vapor Dose condition [F(2,14)=4.34; p<0.05] and of the interaction of factors [F(18,126)=5.08; p<0.0001].

Analysis of the temperature for the 60 minute interval in the female groups confirmed a significant effect of Group [F(1,14)=5.57; p<0.05], of Vapor Inhalation condition [F(3,42)=41.96; p<0.0001] and of the interaction of factors [F(3,42)=3.89; p<0.05] on body temperature. The post hoc analysis confirmed a group difference following inhalation of THC 25-50 mg/mL. The analysis of temperature for the 60 min interval for the male groups likewise confirmed a significant effect of Group [F(1,14)=6.44; p<0.05] and of Vapor Inhalation condition [F(2,28)=31.27; p<0.0001], but not of the interaction of factors. The post hoc test confirmed a significant difference between the groups in the THC 100 mg/mL condition.

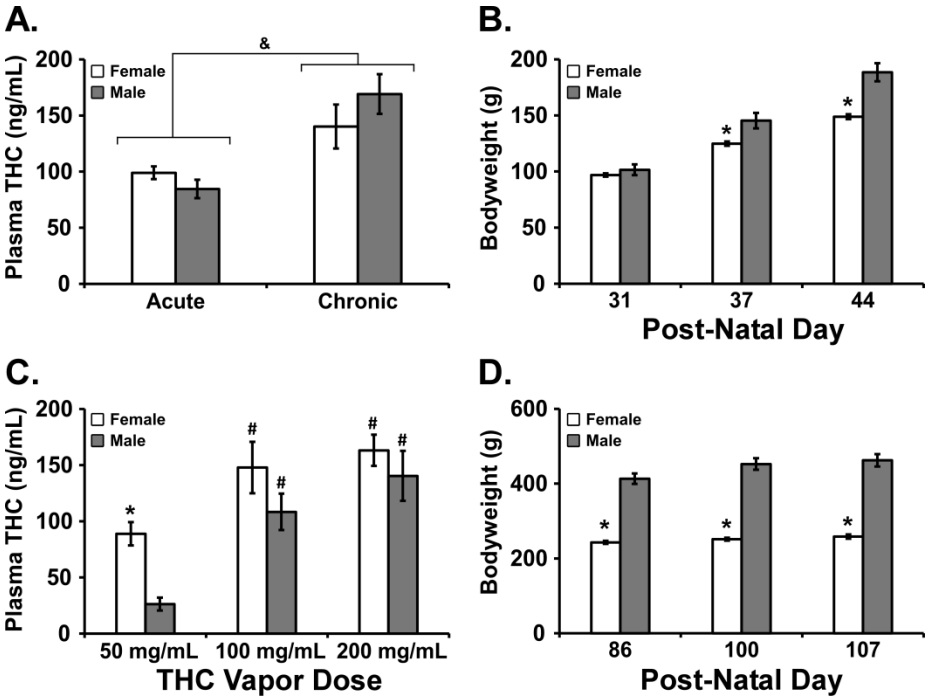

***Figure 5**: A, B) Mean (±SEM) plasma THC levels from female and male rats (N=8 per group) exposed to THC (100 mg/mL) vapor from PND 31-44 (A) or to THC (50-200 mg/mL) from PND 86-107 (C). Corresponding body weights are presented for the adolescent (B) and adult (D) age intervals. A significant sex difference is indicated with *, a difference from the Acute condition across groups is indicated with & and a difference from the 50 mg/mL condition with #.*

Pharmacokinetic experiment

The plasma THC levels did not differ between adolescent male and female rats in either the acute or chronic samples (**Figure 5**). The statistical analysis confirmed a significant effect of Acute versus Chronic experiment phase [F(1,14)=26.64; p=0.0001] without significant effect of Sex or of the interaction of factors. During adult hood, plasma THC levels varied with Vapor Inhalation condition [F(2,26)=20.94; p<0.0001] and

Sex [F(1,13)=7.05; $p<0.05$]. The post hoc test confirmed a significant sex difference at the 50 mg/mL THC dose and a difference from the 50 mg/mL dose after 100 or 200 mg/mL inhalation for each sex. Analysis of the bodyweights confirmed significant differences in the adolescent [PND: F(2,28)=1126; $p<0.0001$; Sex: F(1,14)=10.19; $p<0.01$; Interaction: F(2,28)=71.21; $p<0.0001$] and adult [PND: F(2,28)=183.8; $p<0.0001$; Sex: F(1,14)=143.5; $p<0.0001$; Interaction: F(2,28)=55.82; $p<0.0001$] age ranges. The post hoc tests confirmed that there were significant differences in weight across all three days of the adolescent and adult age ranges for each sex.

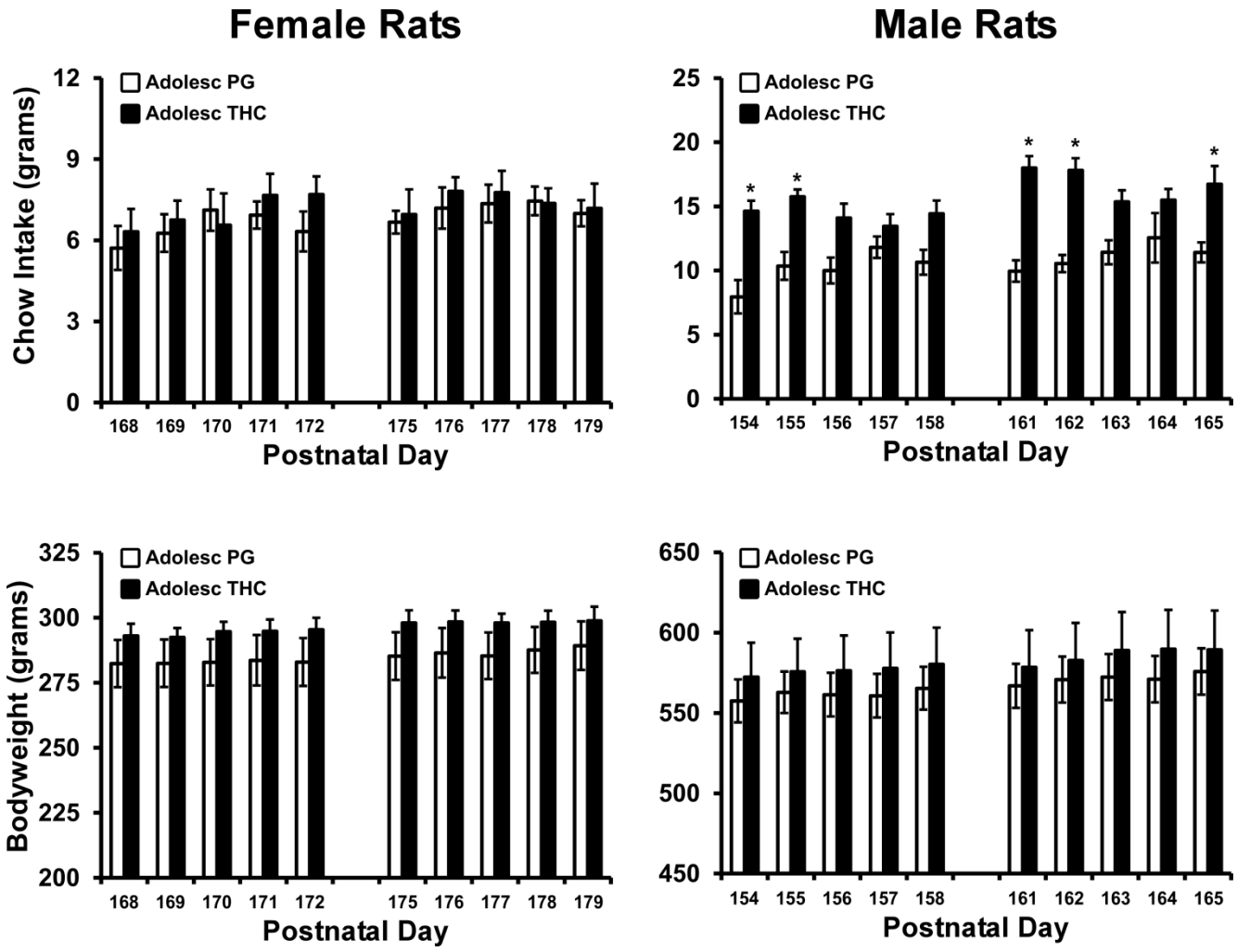

***Figure 6**: Mean (±SEM) chow intake and body weight of female and male rat cohorts exposed to repeated PG or THC during adolescence (N=8 per group except N=7 female/THC) and assessed during adulthood. A significant difference between treatment groups, within sex, is indicated with *.*

Adult food consumption

Feeding was assessed in 6 h sessions during PND 154-158 and PND 161-165 in the males and during PND 105-109, PND 168-172 and PND 175-179 in the female rats (**Figure 6**). One of the female rats from the original THC group was in apparently unrelated ill health and was not included in the PND 168-179 feeding study. The male THC group consumed more chow than the male PG Group with the analysis confirming significant effects of Group [F(1,14)=17.16; p=0.0010], of Day [F(9,126)=4.78; p<0.0001] and of the interaction of Group with Day [F(9,126)=4.97; p<0.0001]. The post hoc test further confirmed the repeated-THC group consumed more food on PND 154, 155, 161, 162 and 165. No significant difference in food intake was confirmed for the female rats in any of the feeding studies. Body weight prior to the session was not significantly different between the Groups for either sex.

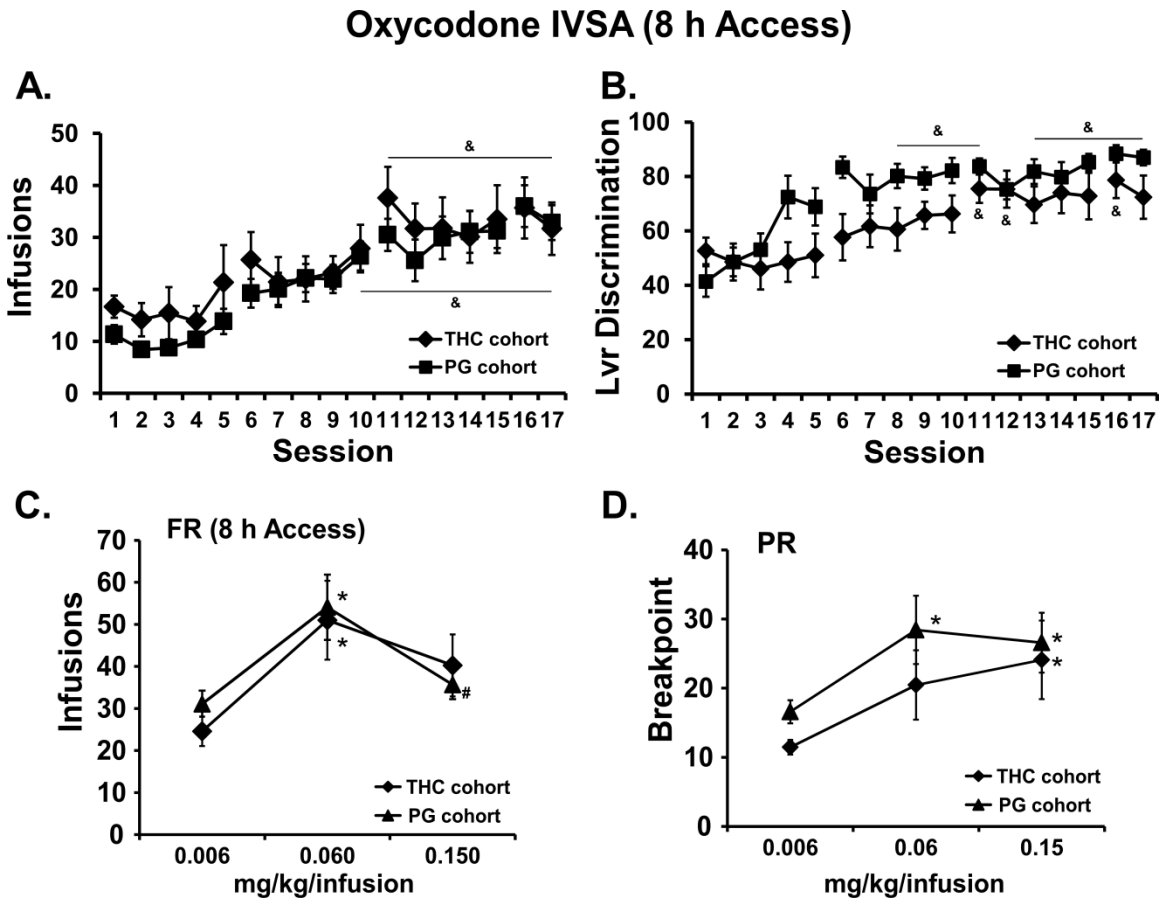

Figure 7. *A) Mean (N=12; ±SEM) infusions and B) percent drug-appropriate lever responding (Lvr Discrimination) for male rats trained to self-administer oxycodone (0.15 mg/kg/inf) within 8 h extended access sessions. C) Mean (THC cohort, N=11; PG cohort, N=12; ±SEM) infusions during self-administration under a FR1 schedule and D) breakpoint values during self-administration under a PR schedule following acute injection of THC (0.006-0.15*

*mg/kg, i.p.). A significant difference from the first session is indicated by &. A significant difference from 0.006 mg/kg/infusion is indicated with * and a difference from the 0.06 is indicated with #.*

Oxycodone self-administration in adulthood

Rats that were exposed to repeated vapor inhalation of either THC or PG vehicle during adolescence significantly escalated their oxycodone self-administration during 17 sessions of acquisition training as adults [$F(16,352)=20.37$; $p<0.0001$]; there was no significant effect of Group (**Figure 7A**). Both groups exhibited a significant increase in appropriate drug-lever responding (lever discrimination) across sessions (**Figure 7B**) as the ANOVA confirmed significant effects of Session [$F(16,352)=13.21$; $p<0.0001$] and of the interaction Group x Session interaction [$F(16, 352)=2.053$; $p<0.01$]. Analysis of oxycodone infusions during dose-substitution experiments under a FR1 schedule confirmed a significant effect of Dose [$F(2, 42)=12.46$; $p<0.0001$] but no effect of Group (**Figure 7C**). Similarly, analysis of the breakpoints under PR confirmed a significant effect of Dose [$F(2, 42)=11.31$; $p=0.0001$] but no effect of Group (**Figure 7D**).

Discussion

This study showed that an e-cigarette based method of Δ^9-tetrahydrocannabinol (THC) inhalation produces tolerance in adolescent rats with repeated exposure. Specifically, adolescent rats of each sex became hypothermic after vapor inhalation of THC, however, repeated inhalation produced rapid tolerance in the female adolescents and a delayed tolerance in both sexes when assessed as adults. This was the case during two weeks (M-F) of twice daily exposure to an inhalation regimen that produced equivalent plasma levels of THC across the sexes (**Figure 5A**), thus confirming the enhanced sensitivity of female rats to the acute development of tolerance given a similar exposure to THC. In addition, tolerance to the thermoregulatory effects of inhaled THC lasted long past the chronic regimen, since adults of each sex from the repeated-THC groups were less sensitive to THC-induced hypothermia compared with their respective repeated-PG control groups when evaluated on PND 86 (**Figure 4**). The tolerance was dose-specific in each sex since an increase in the THC concentration resulted in a similar hypothermia in each adolescent treatment Group. Relatedly, there was a sex difference in the tolerance threshold during adulthood, i.e., the concentration of THC which produced minimal hypothermia in the repeated-THC group but a significant response in the repeated-PG

group. This dose-effect difference may have been due to a sex difference in exposure to THC during adulthood, particularly at lower inhalation dose conditions (**Figure 5**).

The THC-induced temperature change in the repeated-PG groups was lesser on PND 86 compared with PND 31, potentially due to the maturation of the rats and improved thermoregulation. This interpretation is consistent with the fact that body temperature observed 60 min after the start of inhalation on PND 86 was nearly identical to that observed in groups of naïve adult Wistar rats of the respective sexes following 30 min inhalation of THC (100 mg/mL) with no exposure during adolescence (Javadi-Paydar *et al*, 2018). As with our prior studies (Javadi-Paydar *et al*, 2018; Nguyen *et al*, 2016a), spontaneous locomotor behavior was not consistently affected by repeated THC. THC vapor inhalation increased activity rates slightly for about 30 min after inhalation ceased, and some tolerance to this appeared to be present in the female rats during the second week (**Supplemental Figure S3**).

These results are qualitatively congruent with several prior findings using parenteral injections or inhalation of THC. For example, adult female rats (intact or gonadectomized) develop tolerance to THC to a greater extent than males, even with a lower per-injection dose (Wakley *et al*, 2015). Tolerance to the acute locomotor stimulant effects of injected THC developed more rapidly in adolescent female rats compared with adult male rats (Wiley *et al*, 2011) and locomotor tolerance to THC injections was approximately equivalent in adolescent male and female rats after 9 days of repeated THC injection (Wiley and Burston, 2014). Our prior work found female adult rats became tolerant after a repeated THC vapor inhalation regimen that did not produce tolerance in male adults (Nguyen *et al*, 2018). Although females were not assessed, adult male rats exposed to repeated marijuana smoke did not exhibit cross-tolerance to the locomotor suppressing effects of the endogenous cannabinoid agonist anandamide (Bruijnzeel *et al*, 2016). The finding of significantly lower bodyweight in the males during the second treatment week (**Supplemental Figure S2**) is similar to evidence that repeated THC injections during adolescence reduced weight gain in male and female Wistar and Long-Evans rats in a 14 day treatment interval (Keeley *et al*, 2015) and in male Wistar rats during an 8 day repeated-injection study (Sofia *et al*, 1974).

The present findings are likely mechanistically attributable, at least in part, to plasticity in the expression and/or function of the endogenous cannabinoid receptor subtype 1 (CB_1). One prior study of the effects of

repeated adolescent THC exposure found decreased CB_1 receptor expression in the hippocampus of female, but not male rats (Weed *et al*, 2016). Another study found greater CB_1 receptor desensitization in adolescents, compared with adults, and in female adolescents compared with male adolescents, following repeated THC injection. Changes were found in several brain regions including, importantly for the present thermoregulatory data, hypothalamus (Burston *et al*, 2010).

Lasting behavioral effects of adolescent THC exposure are sometimes, but not always, found. Prior studies have identified decreased bodyweight (Rubino *et al*, 2008), impaired spatial working memory (Rubino *et al*, 2009), and greater sensitivity to THC on a learning task (Winsauer *et al*, 2011) in the wake of repeated adolescent THC exposure. In contrast, repeated THC injection (3.2 mg/kg; 8 days) during adolescence did not affect THC place or taste conditioning in adulthood (Wakeford *et al*, 2016). Some prior studies have reported lasting motivational consequences of repeated adolescent THC injection, however the two available studies regarding the effect of repeated adolescent THC exposure on heroin self-administration reached different conclusions. One study in male Wistar rats utilized an injection regimen of twice per day in an escalating regimen of 2.5mg/kg from PND 35–37, 5 mg/kg from PND 38–41 and finally 10 mg/kg from PND42–45 (Stopponi *et al*, 2014). Another study injected male Long-Evan rats with THC (1.5 mg/kg, i.p.) every third day from PND 28-49 (Ellgren *et al*, 2007). Ellgren and colleagues reported increased heroin self-administration in the repeated-THC group when evaluated as adults during acquisition as well as in a post-acquisition dose-substitution procedure under a FR1 contingency. In contrast, Stopponi and colleagues found no differences in the acquisition of intravenous heroin self-administration. The results in the present study are consistent with the latter study and future work could determine if intermittency of exposure, overall dose, specific opioid drug or some other factors are responsible for the differences. Due to a limited number of prior studies and varying THC exposure regimens used it is not possible to draw strong conclusions about the lasting motivational consequences of repeated adolescent exposure at this time. In the a related aspect of the present study, feeding behavior was altered with the repeated-THC males consuming more food in 6 h focal feeding sessions compared with their repeated-PG control group. It will be interesting to determine in future studies if this reflects a motivational change akin to that reported for heroin self-administration by Ellgren and colleagues.

Conclusions

The e-cigarette based vapor-inhalation method is effective for the repeated daily exposure of adolescent rats, generating physiologically significant THC exposure on each session. The exposure regimen selected here produced substantial tolerance in the female adolescents but not in the males, consistent with a sex difference found previously with repeated THC injections. There are also lasting consequences of adolescent exposure on the adult animal including thermoregulatory tolerance in each sex and a feeding phenotype in male rats. This new approach has many advantages including that it avoids the stressor of repeated parenteral injection, entails improved face validity given human cannabis use and may provide improved pharmacokinetic fidelity with human exposure patterns.

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