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Repeated vapor inhalation of Δ9-tetrahydrocannabinol induces tolerance to hypothermia in female rats.

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Abstract

Background and Purpose: Tolerance to the effects of Δ^9-tetrahydrocannabinol (THC) emerges with repeated exposure, although it varies with dose, chronicity and the measure of interest. THC inhalation via an e-cigarette based system induces hypothermic and antinociceptive effects in rats. This study was conducted to determine if tolerance to these effects could be produced with repeated vapor inhalation.

Experimental Approaches: Wistar rats were exposed to 30 minutes of vapor inhalation of the propylene glycol (PG) vehicle and then THC (200 mg/mL in PG) twice per day for four days. Female and male groups were compared for rectal temperature changes and tail-withdrawal latency from a noxious stimulus. A second female group was prepared with radiotelemetry devices for temperature and activity and then exposed to 30 minutes of PG (b.i.d., 4 days) and then THC (100 mg/mL; b.i.d., 5 days). Additional studies evaluated the effects of 4 mg/kg SR141716, AM251 or AM630 i.p. to determine CB_1 and CB_2 receptor contributions.

Key Results: Female, but not male rats developed tolerance to the hypothermic and antinociceptive effects of THC after four days of THC vapor inhalation. The antagonist SR141716 blocked or attenuated antinociceptive effects of acute THC inhalation in male and female rats. Initial hypothermia was not prevented by SR141716 or AM251 but the restoration of normal temperature was accelerated.

Conclusions and Implications: Twice daily THC inhalation induces tolerance in female rats, providing further validation of the method. Blockade of the CB_1 receptor shortens the maintenance of hypothermia and blocks antinociception after THC inhalation.

Key Words: cannabis; marijuana; hypothermia; nociception; tolerance; e-cigarette

Abbreviations: PG, propylene glycol; SR141716 (SR), 5-(4-Chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-*N*-(piperidin-1-yl)-1*H*-pyrazole-3-carboxamide; AM251, *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; AM630, 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)methanone; THC; Δ^9tetrahydrocannabinol;

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Introduction

Increasing use of e-cigarette type devices by human cannabis users (Mammen, Rehm & Rueda, 2016; Morean, Kong, Camenga, Cavallo & Krishnan-Sarin, 2015; Morean, Lipshie, Josephson & Foster, 2017), spurs the development of pre-clinical models. Recent studies showed that intrapulmonary delivery of THC using an e-cigarette based system results in a robust and dose-dependent hypothermia in male and female rats (Nguyen et al., 2016). A similar system results in hypothermia in mice following inhalation exposure to synthetic cannabinoid agonists (Lefever et al., 2017). Such systems also induces locomotor stimulation after inhalation of methamphetamine, mephedrone or 3,4-methylenedioxypyrovalerone (Nguyen, Aarde, Cole, Vandewater, Grant & Taffe, 2016) and can produce escalated self-administration of sufentanil to the point of dependence (Vendruscolo et al., 2017). Prior inhalation cannabinoid studies did not attempt to provide any evidence of plasticity of the cannabinoid-typical behavioral or physiological effects that attend repeated dosing. Repeated exposure to THC via parenteral injection produces tolerance to the acute effects in male and female laboratory animals such as mice (Anderson, Jackson, Chesher & Malor, 1975; Fan, Compton, Ward, Melvin & Martin, 1994), rats (Jarbe, 1978; Taylor & Fennessy, 1978; Wiley & Burston, 2014) or monkeys (Ginsburg, Hruba, Zaki, Javors & McMahon, 2014; Smith, Almirez, Berenberg & Asch, 1983; Winsauer et al., 2011). Thus it is of significant interest to determine if repeated exposure to THC via e-cigarette type inhalation is capable of producing tolerance in rats to further validate this model.

A substantial and sustained decrease in body temperature and a reduction in sensitivity to a noxious stimulus (antinociception) are two major indicators of cannabinoid-like activity in laboratory rodents (Wiley, Marusich & Huffman, 2014). These effects appear to mostly be mediated via the CB_1 receptor since the CB_1 antagonist/inverse agonists SR141716 (Rimonabant; SR) and AM251 have been shown to block them. Prior studies from this laboratory found that SR, administered 15 minutes prior to intraperitoneal injection of THC blocked the body temperature decreases, and tail-flick latency increases (Taffe, Creehan & Vandewater, 2015). This is consistent with prior reports that SR pretreatment attenuated a prolactin response to THC (Fernandez-Ruiz, Munoz, Romero, Villanua, Makriyannis & Ramos, 1997) and hypothermia caused by injection of the CB1 full agonist WIN 55,212-2 in mice (Son et al., 2010). SR also blocked antinociceptive, hypothermic and hypolocomotor effects of inhaled THC in male Swiss Webster mice (Marshell et al., 2014). SR and AM251 prevented hypothermic and antinociceptive effects of THC, i.p., but failed to reverse hypolocomotor effects, in male C57BL/6J mice (McMahon & Koek, 2007). Similar effects have also been demonstrated following inhalation of THC (or cannabis smoke) but efficacy may be more variable. SR blocked hypolocomotive effects of marijuana smoke inhalation in rats (Bruijnzeel et al., 2016) and fully blocked antinociception caused by aerosolized THC, but only partially blocked the antinociceptive effects of cannabis smoke inhalation, in male ICR mice (Lichtman et al., 2000; Lichtman, Poklis, Poklis, Wilson & Martin, 2001). Catalepsy caused by marijuana smoke inhlation was not blocked by SR pretreatment in the latter study. Interestingly, SR fully reversed the antinociceptive, hypothermic and cataleptic effects of THC when administered intravenously in the same behavioral model(Lichtman, Poklis, Poklis, Wilson & Martin, 2001). Finally, SR failed to reverse the effects of THC, i.m., on operant responding in monkeys and only partially reversed hypothermia observed 2 h after injection (McMahon, Amin & France, 2005).

Further explication of the mediation of the effects of THC inhalation via the e-cigarette model by CB_1 activity is needed. It was shown that SR blocked antinociceptive effects of the inhalation of THC (Nguyen et al., 2016), but no evidence on the effect of antagonist pre-treatment on THC-induced hypothermia was included in that study. As was briefly reviewed above, SR does not always fully block effects of THC and this may depend on outcome measure, species, THC dose or other as yet unexplicated factors. This study was therefore designed to determine if the body temperature response to, and antinociceptive effects of, the inhalation of THC via e-cigarette type technology are attenuated by repeated, twice-daily exposure in male or female rats. Additional studies were conducted to determine if the hypothermic or antinociceptive responses could be blocked by pre-treatment with CB_1 antagonist/inverse agonists. As humans increasingly ingest THC via e-cigarette technology it become increasingly important to develop pre-clinical models that are capable of evaluating the consequences of this route of administration.

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Methods

Animals: Male (N=16) and female (N=23) Wistar (Charles River, New York) and male Sprague-Dawley (Harlan, Livermore, CA) (N=8) rats were housed in humidity and temperature-controlled (23±2 °C) vivaria on 12:12 hour light:dark cycles. Rats had *ad libitum* access to food and water in their home cages. All experiments were performed in the rats' scotophase. Rats initially entered the laboratory at 10-11 weeks of age. All procedures were conducted under protocols approved by the Institutional Care and Use Committee of The Scripps Research Institute.

***Radiotelemetry*:** A group of female rats (N=8) were implanted with sterile radiotelemetry transmitters (Data Sciences International, St Paul, MN; TA-F40) in the abdominal cavity as previously described (Taffe, Creehan & Vandewater, 2015; Wright et al., 2012). Animals were evaluated in clean standard plastic homecages (thin layer of bedding) in a dark testing room, separate from the vivarium, during the (vivarium) dark cycle. 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, Huang, Creehan, Dickerson & Taffe, 2013; Miller et al., 2013). Test sessions started with a 15 minute interval to ensure data collection then a 15 minute interval for baseline temperature and activity values followed by initiation of vapor sessions. The 15 minute baseline was omitted for the studies with intraperitoneal injection of THC due to the delayed onset of hypothermia (Nguyen et al., 2016; Taffe, Creehan & Vandewater, 2015). Pre-treatment drugs or vehicle for the antagonist studies were injected prior to the 15 minute baseline interval. Since data collection was automated, the investigator was not blinded to treatment condition for these assays.

Materials: Δ^9-tetrahydrocannabinol (THC) was administered by vapor inhalation with doses described by the concentration in the propylene glycol (PG) vehicle and duration of inhalation. THC was also administered intraperitoneally in a dose of 10 mg/kg. SR141716 (Rimonabant; **SR**), AM251 or AM630 were administered intraperitoneally in a dose of 4 mg/kg. For injection, THC, SR, AM251 or AM630 were suspended in a vehicle of 95% ethanol, Cremophor EL and saline in a 1:1:8 ratio. The THC was provided by the U.S. National Institute on Drug Abuse; SR141716 was obtained from ApexBio (New Delhi, Delhi, India; Distributor: Fisher Scientific, Pittsburgh, PA, USA); AM251 and AM630 were obtained from Tocris Bioscience (Avonmouth, Bristol, UK; Distributor: Fisher Scientific, Pittsburgh, PA, USA).

Inhalation Apparatus: 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 as has been previously described (Nguyen et al, 2016a; Nguyen et al, 2016b). 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 MedPC IV software (Med Associates, St. Albans, VT USA). 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.

Nociception Assay: Tail-withdrawal antinociception was assessed using a water bath (Bransonic® CPXH Ultrasonic Baths, Danbury, CT) maintained at 52°C. The latency to withdraw the tail was measured using a stopwatch, and a cutoff of 15 seconds was used to avoid any possible tissue damage (Wakley and Craft, 2011; Wakley et al, 2014). Tail-withdrawal was assessed 35, 60 and 120 minutes after the initiation of inhalation. The person performing the assay was blinded to the treatment condition for a given subject.

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Experiments

Study 1: Sex Comparison

The initial study was conducted in groups of un-operated male (N=8; 17 weeks of age, 463.5 (SEM 14.4) g bodyweight at start of this study) and female (N=8; 17 weeks of age, 233.4 (SEM 5.1) g bodyweight at start of this study) Wistar rats. The body temperature was determined by rectal measurement with a lubricated thermistor (VWR Traceable™ Digital Thermometer) as previously described (Gilpin, Wright, Dickinson, Vandewater, Price & Taffe, 2011). The person performing the rectal temperature and nociception assays was blinded as to the treatment condition for a given subject.

Inhalation sessions were 30 minutes in duration with four vapor puffs delivered every 5 minutes with a 5:15 h interval between session initiations on each repeated-exposure day. The THC concentration was 200 mg/ml in this study. These animals had been initially evaluated with parenteral injection of THC (0, 10, 20, 20 mg/kg i.p.) in the stated (ascending) order with 2-3 day intervals between test days completed six weeks prior to the start of the chronic vapor inhalation experiment (not shown). For the current study, animals were first evaluated for temperature and nociceptive responses after one PG-inhalation session. Four days of repeated THC was initiated the following week. For the repeated dosing, rats were exposed to vapor inhalation for 4 sequential days with twice-daily sessions (4 h interval between session initiations each day). Temperature and antinociceptive effects of THC inhalation were assessed after the first and seventh sessions.

Study 2: Telemetry Assessment

The second study was conducted with a group of female rats (N=8; 11 weeks of age, 199.8 (SEM 2.4) g bodyweight at start of this study) implanted with radiotelemetric devices. This provided the ability to monitor temperature responses without the handling stress of repeated rectal assessment and facilitated a more precisely determined timecourse of tolerance as it developed across the 7 sessions in female rats. Males were not included in this study due to the lack of tolerance observed in Study 1. Inhalation sessions were 30 minutes in duration with four vapor puffs delivered every 5 minutes. The THC concentration was reduced to 100 mg/ml in this study, compared with Study 1, to determine if this concentration was sufficient to produce tolerance to THC-induced hypothermia. In the first week, rats received four days of twice-daily inhalation of the PG vehicle (5:15 h interval between session initiations on each day). The following week, rats received five days of twice daily inhalation of THC (100 mg/mL), again with 5:15 h between sessions. Additional THC-inhalation sessions were conducted one and two weeks after the final session of the repeated-THC week.

Study 3: Pharmacological mechanisms of THC-induced hypothermia

The third study was conducted in the radiotelemetry implanted group of female rats (starting 20 days after the last THC-inhalation in Study 2) to determine if the hypothermic effects of THC inhalation were mediated by CB_1 and/or CB_2 receptors using pretreatment with 4 mg/kg, i.p. of the CB_1 antagonists/inverse agonists SR141716 and AM251 and CB_2 antagonist/inverse agonist AM630. In these studies, the THC doses were administered no more frequently than a 7 day interval and the order of the pre-treatment / inhalation conditions was randomized across the group within each study.

Study 4: THC i.p. validation

This study was conducted in the same group of telemetry-implanted female rats to determine the effect of SR141716 and AM251 on the hypothermic effect of parenterally injected THC (10 mg/kg, i.p.).

Study 5: SR in male rats

This study was conducted in a group of male (N=8; 19 weeks of age, 440 (SEM 7.9) g bodyweight at start of this study) Sprague-Dawley rats implanted with radiotelemetric devices used in the studies reported in a prior report on the efficacy of the vapor inhalation model (Nguyen et al., 2016). The present study was conducted after Experiment 1 in that report (which evaluated the effect of vapor inhalation of THC (200 mg/mL) for 10, 20 or 30 minutes in otherwise naïve animals). In this study, animals received either the vehicle or SR141716 (4 mg/kg, i.p.) 15 minutes before the initiation of THC (200 mg/mL) inhalation for 20 minutes.

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Study 6: Antinociception

This study was conducted in groups of male (N=8) and female (N=7) Wistar rats that had previously been exposed to THC vapor inhalation (12.5-100 mg/mL; 30 minutes) to determine plasma levels of THC with dosing no more frequently than a 2 week interval necessary for recovery of blood volume after repeated sampling (Diehl et al., 2001). These groups were 19 weeks of age at the start of the present study. The female group was originally N=8 but one animal was lost to the study during surgical implantation of an intravenous catheter. For the present study, tail-withdrawal was assessed 35, 60 and 120 minutes after initiation of vapor inhalation of PG or THC (100 mg/mL for 30 minutes with 4 puffs every 5 minutes) with SR administered 15 minutes prior to PG (4 mg/kg SR, i.p.) or THC (0, 4 mg/kg SR, i.p.). Treatment order of these three conditions was balanced across both male and female groups, with the vapor inhalation conditions run in same-sex pairs.

Data Analysis: The data and statistical analysis comply with recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015).The telemeterized body temperature and activity rate (counts per minute) were collected on a 5-minute schedule in telemetry studies but are expressed as 30 minute averages for analysis (baseline values are from 15 minute intervals). The time courses for data collection are expressed relative to the THC injection time or the initiation of vapor inhalation and times in the figures refer to the end of the interval (e.g. “60 minutes” reflects the data collected from 35 to 60 minutes, inclusive). Any missing temperature values were interpolated from the values before and after the lost time point. Activity rate values were not interpolated because 5-minute to 5-minute values can change dramatically, thus there is no justification for interpolating. Group sizes were determined initially with a power analysis of similar data collected previously in the laboratory. Data (rectal temperature, tail-withdrawal latency, telemeterized temperature and activity measures) were analyzed with two way Analysis of Variance (ANOVA) including repeated measures factors for the Drug treatment condition and the Time after vapor initiation or injection. Any significant main effects were followed with post-hoc analysis using Tukey, Sidak or Dunnett correction as specified. All analysis used Prism 7 for Windows (v. 7.03; GraphPad Software, Inc, San Diego CA).

Results

Study 1: Sex Comparison

The inhalation of THC (200 mg/mL in the PG; 30 minute exposure) reduced body temperature and increased tail-withdrawal latency in male and female rats (**Figure 1)**. Statistical analysis of the female rectal temperature confirmed significant effects of Time [$F (3, 21) = 19.85$; $P<0.0001$], Drug treatment condition [$F (2, 14) = 58.15$; $P<0.0001$] and the interaction of factors [$F (6, 42) = 14.08$; $P<0.0001$]. The Tukey post-hoc test confirmed that rectal temperature was significantly lower after the first THC session compared with the PG session (30-120 minutes post-initiation) or with the seventh THC session (30-60 minutes post-initiation), and lower after the seventh THC session relative to vehicle (30 minutes). Statistical analysis of the male rectal temperature similarly confirmed main effects of Time [$F (3, 21) = 6.96$; $P<0.005$], Drug treatment condition [$F (2, 14) = 20.91$; $P<0.0001$] and the interaction of factors [$F (6, 42) = 4.69$; $P<0.005$]. In this case the Tukey post-hoc analysis confirmed that rectal temperature was significantly lower after the first (30-60 minutes post-initiation) and seventh (30-240 minutes post-initiation) THC sessions compared with the PG session but did not significantly differ between the two THC sessions.

THC inhalation also increased tail-withdrawal latencies in both male and female groups (**Figure 1**). The analysis of the female withdrawal latencies confirmed significant effects of Time [$F (3, 21) = 10.79$; $P<0.0005$], Drug treatment condition [$F (2, 14) = 10.5$; $P<0.005$] and the interaction of factors [$F (6, 42) = 2.51$; $P<0.05$]. The Tukey post-hoc confirmed that withdrawal latency was longer in the first THC session compared with the PG session (30-120 minutes post-initiation) or the seventh THC session (30 minutes post-initiation) and shorter after PG inhalation relative to the seventh THC session (120-240

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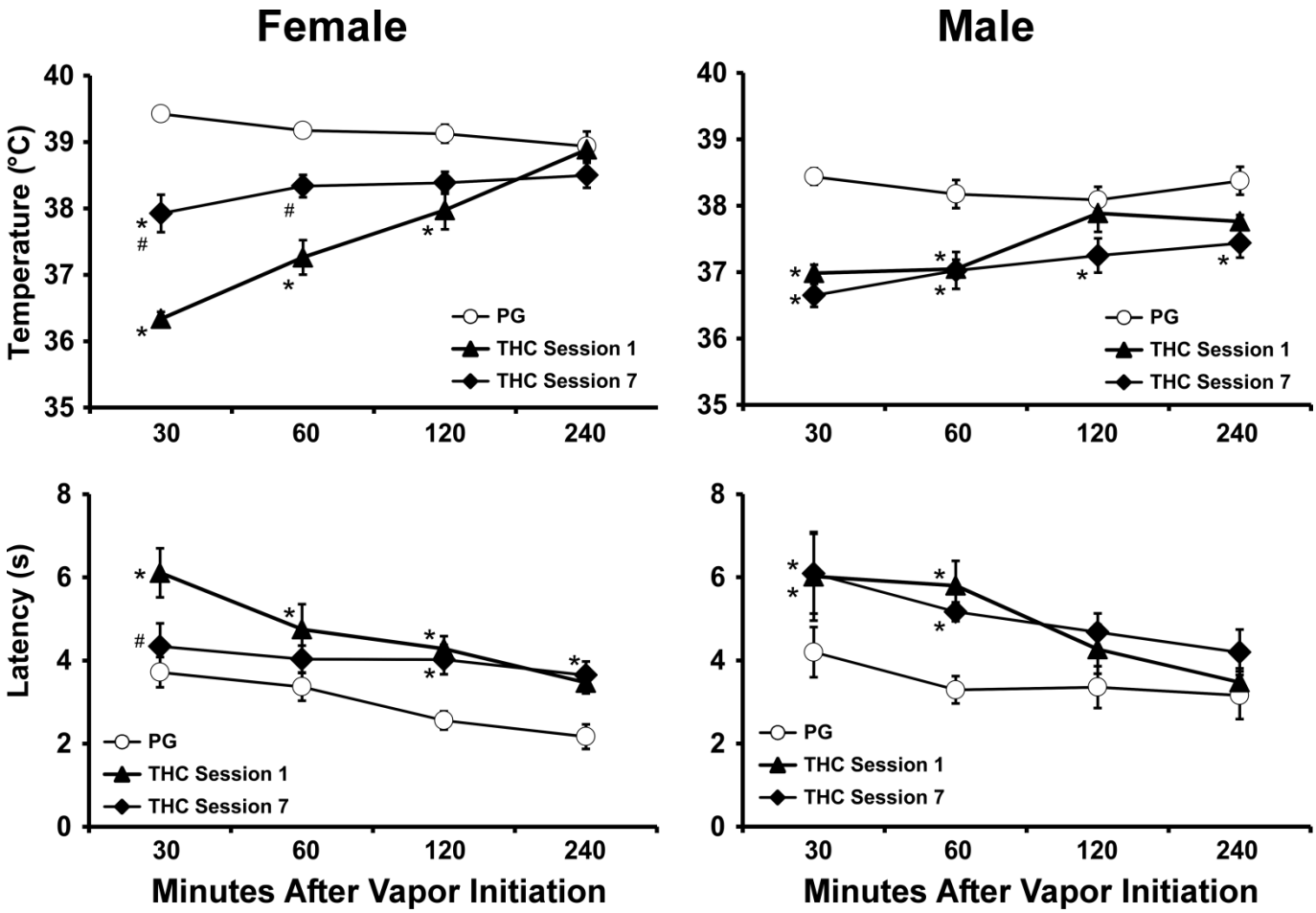

***Figure 1**: Mean (N=8 per group; ±SEM) rectal temperature (upper panels) and tail-withdrawal latency (lower panels) for male and female rats after inhalation of the PG vehicle or THC (200 mg/mL) on the 1st or 7th repeated session. Significant differences from the PG condition are indicated with * and from the first THC Session with #.*

minutes). Analysis of the male rat tail-withdrawal latency confirmed main effects of time [F (3, 21) = 6.13; P<0.005] and drug treatment condition [F (2, 14) = 4.6; P<0.05] but not of the interaction. The Tukeypost-hoc on the marginal means confirmed that withdrawal latency was significantly longer in the seventh THC session compared with vehicle but did not differ between the first and seventh THC sessions.

Study 2: Telemetry Assessment

Naïve female rats first exposed to four days of twice-daily inhalation of PG (30 minutes) exhibited no acute reduction in body temperature post-initiation of vapor as is shown in **Figure 2**. In contrast body temperature was acutely reduced by inhalation of THC (100 mg/mL; 30 minutes) during the repeated-THC week. The analysis of variance was conducted on 30 minute bins and included the first daily session for all nine treatment days. The ANOVA confirmed significant effects of Session [F (8, 56) = 17; P<0.0001], of Time after vapor initiation [F (9, 63) = 19.7; P<0.0001] and of the interaction of factors [F (72, 504) = 9.98; P<0.0001]. The Tukey post-hoc test (see Figure 2 for details) confirmed that there was no hypothermia relative to baseline in the first 180 minutes after the initiation of inhalation for any of the PG sessions. In contrast the inhalation of THC induced a consistent hypothermia relative to the pre-inhalation baseline up to 210 minutes after initiation for all five days. Similarly, the post-hoc confirmed that body temperature was lower compared with all four PG sessions 60 minutes after initiation of vapor in all five THC sessions. This continued for up to 180 minutes after initiation on the first two THC sessions. THC-associated hypothermia was significantly attenuated in initial magnitude on THC days 4 and 5 compared with the first three days.

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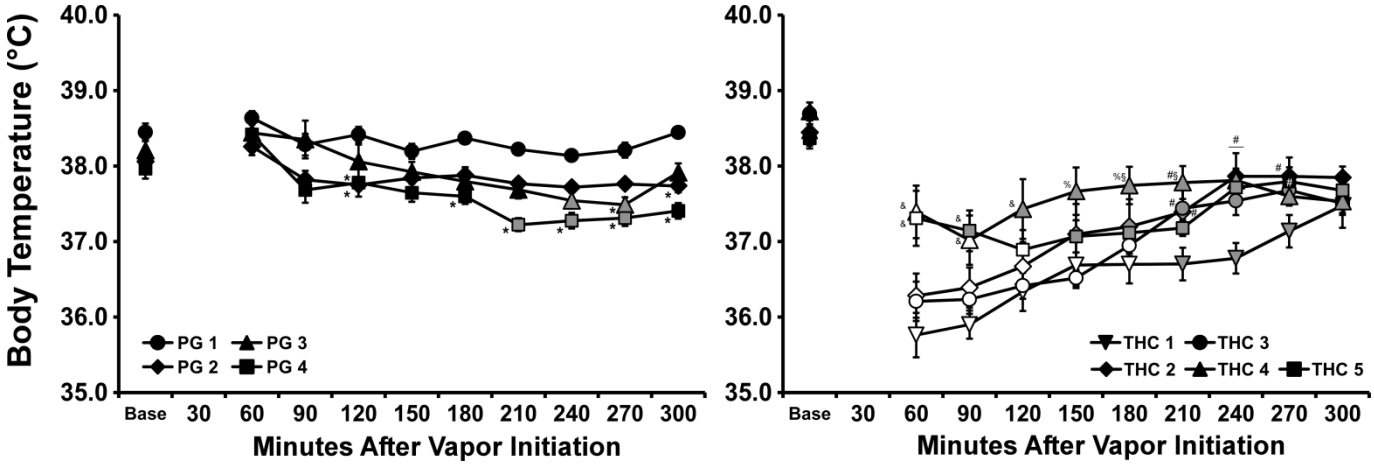

***Figure 2**: Mean (N=8; ±SEM) body temperature before (Base= baseline) and after vapor inhalation of PG or THC (100 mg/mL) for 30 minutes. Difference from PG day 1 by *; difference from THC day 1 by #; significant difference from THC days 1-3 by &; significant difference from THC days 1 and 3 by %; significant difference between THC 4and THC 5 by §; a bar indicates all other days differ.*

Study 3: Pharmacological mechanisms of THC-induced hypothermia

Additional studies were conducted in the group of female Wistar rats used in Study 2 to probe dependence of the THC-induced hypothermia on the CB_1 or CB_2 endocannabinoid receptor subtypes. There were two additional THC sessions conducted 1 and 2 weeks after the repeated THC week (summarized below) and these pharmacological treatments were conducted 20 days after the last THC session. For all these experiments the rats were exposed to THC no more frequently than a 7 day interval. In the first experiment (**Figure 3A**), SR141716 (4 mg/kg, i.p.) or the vehicle was administered 15 minutes prior to start of vapor inhalation of PG or 100 mg/mL THC for 30 minutes. The four conditions were conducted in randomized order and the ANOVA confirmed a significant effect of Time after vapor initiation [F (8, 56) = 7.38; P<0.0001], of Treatment condition [F (3, 21) = 8.44; P<0.001] and of the interaction [F (24, 168) = 8.08; P<0.0001]. The Tukey post-hoc test confirmed a significant difference between Vehicle and SR pretreatment conditions from 90-150 minutes after THC vapor initiation and 60 minutes after PG vapor initiation.

In the next experiment (**Figure 3B**) AM251 (4 mg/kg, i.p.) or the vehicle was administered 15 minutes prior to start of vapor inhalation of 100 mg/mL THC for 30 minutes. The two conditions were conducted in randomized order. The ANOVA confirmed a significant effect of Time after vapor initiation [F (8, 56) = 11.65; P<0.0001], of Treatment condition [F (1, 7) = 18.73; P<0.005] and of the interaction of factors [F (8, 56) = 6.38; P<0.0001]. The Tukey post-hoc test confirmed a significant difference between Vehicle and AM251 pre-treatment for 60-180 minutes after the start of vapor. Finally, AM630 (4 mg/kg, i.p.) or the vehicle was administered 15 minutes prior to start of vapor inhalation of PG or 100 mg/mL THC for 30 minutes (**Figure 3C**). The four conditions were conducted in randomized order and the ANOVA confirmed a significant effect of Time after vapor initiation [F (8, 56) = 4.59; P<0.0005], of Treatment condition [F (3, 21) = 23.62; P<0.0001] and of the interaction [F (24, 168) = 4.24; P<0.0001]. The Tukey post-hoc test confirmed that body temperature was significantly lower compared with both the baseline and the respective PG inhalation condition for Veh-THC (30-180 minutes after initation of vapor) and for AM630-THC (30-120 minutes). Body temperature was significantly lower after THC inhalation compared the respective PG conditions up to 240 minutes after the start of inhalation.

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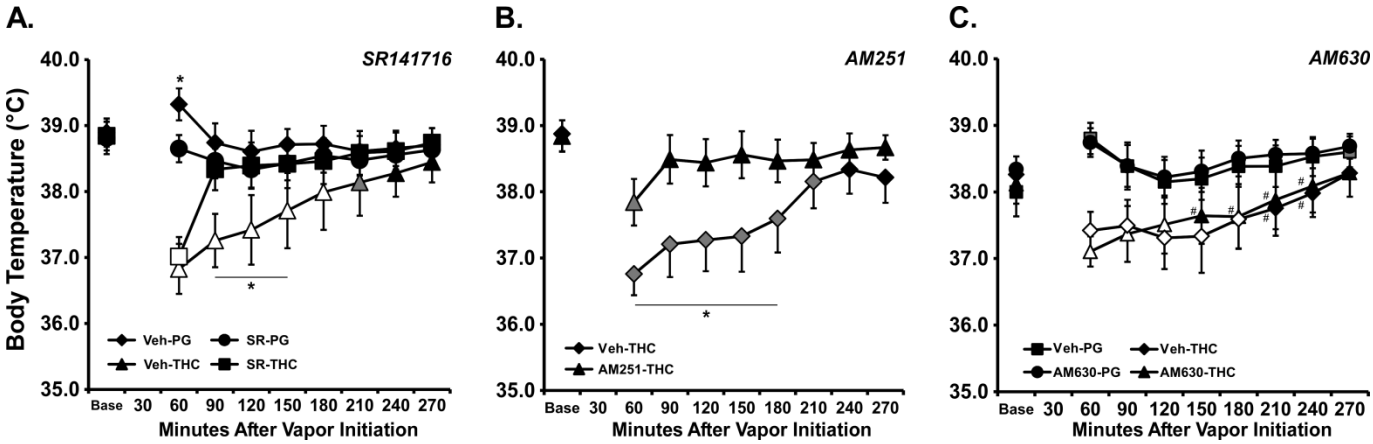

Figure 3: *Mean (N=8; ±SEM) body temperature before (Base= baseline) and after vapor inhalation of PG or THC (100 mg/mL) for 30 minutes. Shaded symbols indicate a significant difference from the baseline value, open symbols indicate a significant difference from the baseline and the respective PG inhalation condition (when run). A significant difference from the respective PG condition only is indicated with #. A significant difference between vehicle and antagonist pre-treatment is indicated with *.*

An analysis of all of the THC vapor inhalation studies in this group of animals was conducted to assess the stability of THC-induced hypothermia observed 60 minutes after vapor initiation across the intermittent challenges conducted subsequent to the repeated THC week. This included follow-up studies of THC 100 mg/mL for 30 minutes conducted one and two weeks after Session 5 and the Vehicle-THC conditions for the Study 3 investigations (**Figure 4**). The ANOVA confirmed a significant effect of Time after vapor initiation [F (1, 7) = 55.18; P=0.0001], of Treatment condition [F (13, 91) = 4.73; P<0.0001] and of the interaction [F (13, 91) = 25.91; P<0.0001]. The Tukey post-hoc test confirmed that significant differences from baseline were observed for all THC inhalation sessions. The Dunnett post-hoc confirmed that body temperature 60 minutes after the start of inhalation differed from the 5th THC session in all repeated-PG sessions and the first three repeated-THC sessions but remained unchanged throughout the subsequent studies.

<u>Study 4: THC i.p. validation</u>

This study was conducted after the AM630 study in the female Wistar group. For this study animals were injected with THC (0, 10 mg/kg i.p.) with the vehicle, SR141716 (4 mg/kg, i.p.) or AM251 (4 mg/kg, i.p.) administered 15 minutes prior to THC. The analysis of all six treatment conditions confirmed a significant effect of Time post-injection [F (9, 63) = 22.18; P<0.0001], of Drug treatment condition [F (5, 35) = 4.15; P<0.005] and of the interaction [F (45, 315) = 3.37; P<0.0001]. The Tukey post-hoc test confirmed that temperature was lower than baseline and the respective time points after vehicle-vehicle injection when THC was preceded by the vehicle (30-270 minutes post-injection), when THC was preceded by AM251 (60-270 minutes post-injection) and when the vehicle was preceded by AM251 (90-120 minutes post-injection). Temperature was lower in the Veh-THC condition compared with the SR-THC treatment from 30-270

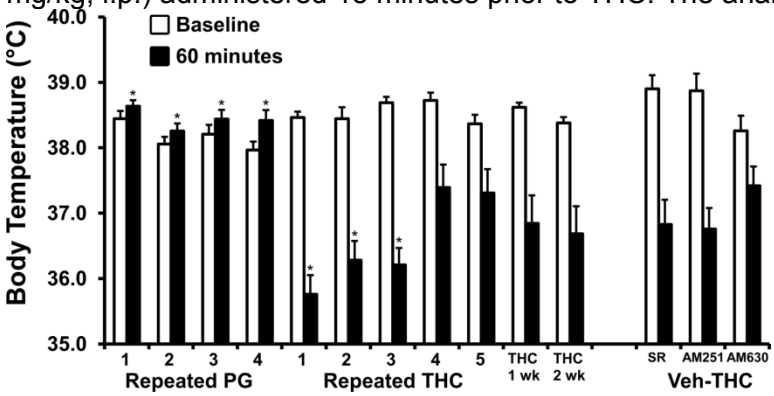

Figure 4*: Mean (N=8; ±SEM) body temperature before (Base= baseline) and 60 minutes after initiation of vapor inhalation of PG or THC (100 mg/mL) for 30 minutes.A significant difference from the repeated THC session 5 is indicated with *.*

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minutes after the THC injection. No differences were confirmed for the SR-Veh/SR-THC or AM251-Veh/AM251-THC pairs of conditions.

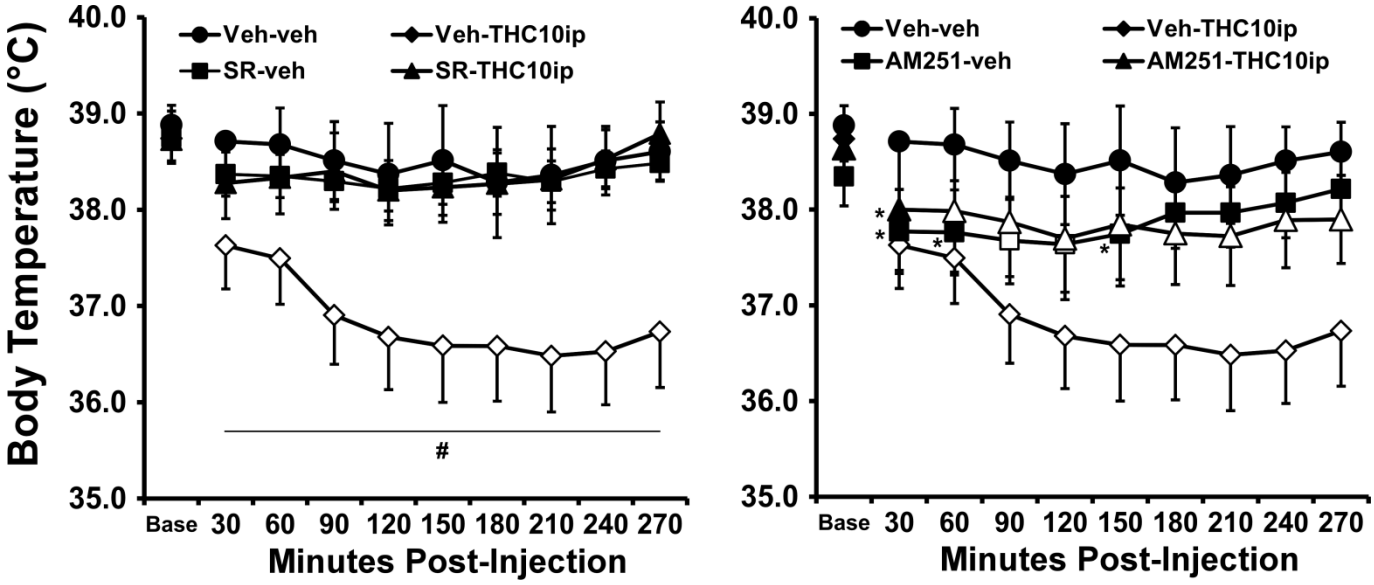

Figure 5*: Mean (N=8; ±SEM) body temperature after i.p. injection of THC (10 mg/kg) or the Vehicle following pre-treatment with left) SR141716 (4 mg/kg, i.p.) or right) AM251 (4 mg/kg, i.p.). The Vehicle-Vehicle and Vehicle-THC conditions are depicted in both panels for comparison. Shaded symbols indicate a significant difference from the baseline value, open symbols indicate a significant difference from the baseline and the vehicle condition. A significant difference from the Vehicle-Vehicle condition is indicated with * and between Veh and SR pre-treatment THC conditions with #.*

Study 5: SR in Male Sprague-Dawley rats

Treatment of male Sprague-Dawley rats (N=8) with 4 mg/kg SR141716, i.p.,15 minutes prior to THC (200 mg/L) for 20 minutes altered the course of the hypothermic response (**Figure 6**). Analysis confirmed significant effects of Time post-initiation [F (6, 42) = 29.65; P<0.0001] and of the interaction of Time post-initiation with the pre-treatment condition [F (6, 42) = 3.57; P<0.01]. The Tukey post-hoc test confirmed that temperature was significantly lower than both pre-inhalation time points for Veh + THC (30-90 minutes after vapor initiation) and for SR + THC (30-60 minutes after vapor initiation. The post-hoc test further confirmed that body temperature was higher 90 minutes after vapor initiation when SR was administered compared with the vehicle.

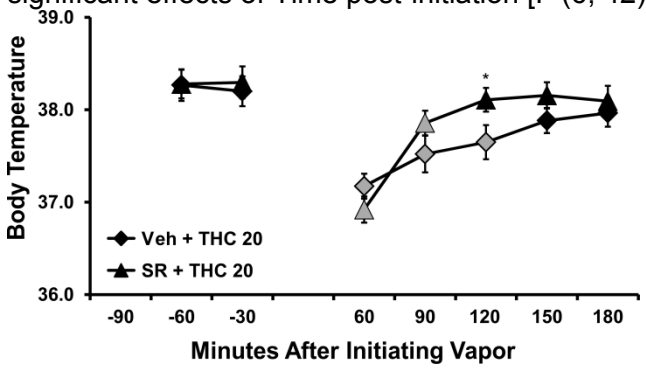

*Figure 6: Mean (N=8; ±SEM) body temperature before and after vapor inhalation of PG or THC (200 mg/mL) for 20 minutes. Shaded symbols indicate a significant difference from the baseline value and a significant difference between SR141716 (SR) pre-treatment conditions is indicated with *.*

Study 6: Antinociception

The analysis of the female rat data confirmed a significant effect of Drug treatment condition [F (2, 12) = 23.01; P<0.0001] but not of Time after vapor initiation or of the interation of factors. Post-hoc analysis of the marginal means confirmed that tail-withdrawal latency differed significantly across all three treatment conditions. The analysis of the male rat data confirmed a significant effect of Drug

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treatment condition [F (2, 14) = 13.54; P=0.0005], of Time after vapor initiation [F (2, 14) = 5.85; P=0.0142] but not of the interation of factors. The Tukey post-hoc analysis confirmed that tail-withdrawal latency was significantly longer after the Veh-THC condition compared with the SR-PG (35-60 minutes after initiation of inhalation) and SR-THC (35 minutes post-initiation) conditions.

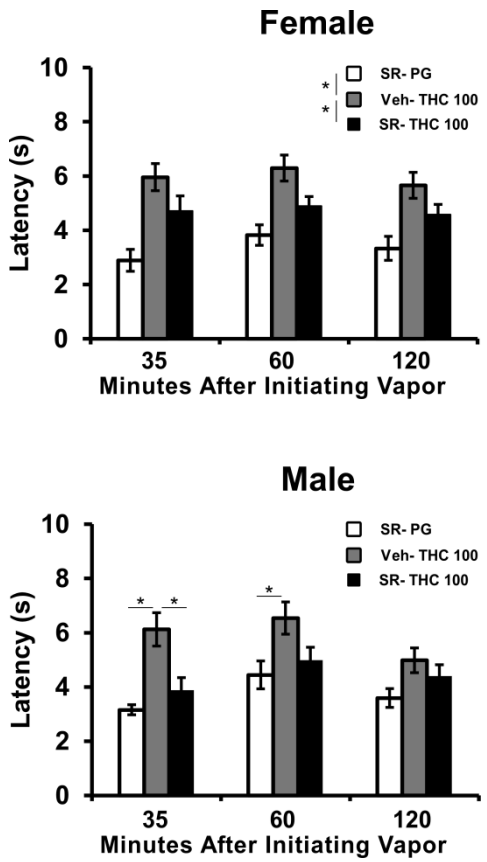

*Figure 7: Mean tail-withdrawal latencies for female (N=7; ±SEM) and male (N=8; ±SEM) rats. Significant differences between pairs of conditions or the marginal means are indicated with *.*

Discussion

This study is the first to show that repeated inhalation of THC vapor using an e-cigarette approach induces tolerance to hypothermia in rats. Significant tolerance was observed only following the 7th session of a twice daily regimen in female rats, and this was the case when the inhalation drug concentration was either 200 or 100 mg/mL THC in the PG. Similar to what has been previously shown (Javadi-Paydar, Nguyen, Grant, Vandewater, Cole & Taffe, 2017; Nguyen et al., 2016), no substantial tolerance was observed when repeated THC inhalation sessions are spaced by at least 7 days, since the magnitude of the body temperature effect was similar to Sessions 4-5 of Study 2 in two additional THC inhalation sessions conducted at weekly intervals after the chronic THC week as well as during all of the vehicle pretreatment / THC inhalation conditions of Study 3 (**Figure 4**). This study also confirms that the hypothermic and antinociceptive responses to inhaled THC are mediated by, at least in part, CB_1 receptor function. A novel and unexpected outcome was that the initial hypothermia generated by THC inhalation was not blocked by SR or AM251 as is the case for parenteral injection of THC.

These studies define some conditions under which tolerance to the thermoregulatory and antinociceptive effects of vapor inhalation of THC are produced. In female rats, four days of twice daily inhalation for 30 minutes is sufficient to produce significant tolerance. The same regimen did not produce thermoregulatory or nociceptive tolerance in males over the same time course. It is possible that this is due to a difference in the THC dose reaching the brains of male versus female animals, but this is unlikely due to the approximately similar magnitude of effects on nociception and thermoregulation that were produced. It is more likely that this outcome reflects a sex difference in the development of tolerance to the effects of THC. Prior work has shown that female mice develop tolerance to locomotor stimulant effects of THC (i.p.) under conditions under which males do not (Wiley, 2003), that female rats develop tolerance to THC-induced errors on a learning task more slowly than males during chronic injection of 10 mg/kg THC, i.p., (Weed, Filipeanu, Ketchum & Winsauer, 2016) and that female rats develop a greater degree of nociceptive tolerance to THC even when the repeated dose is only 71% as large as the male dose (Wakley, Wiley & Craft, 2014). The study also demonstrated, as in our prior reports (Javadi-Paydar, Nguyen, Grant, Vandewater, Cole & Taffe, 2017; Nguyen et al., 2016), that so long as intervals of at least 7 days are maintained between THC administration sessions, there is no detectable plasticity of the hypothermic response.

It was previously shown that the antinociceptive effect of inhalation THC vapor can be blocked by SR141716 (SR; 4 mg/kg, i.p.) in male Wistar rats (Nguyen et al., 2016). The present study replicates this effect in male rats and found a partial attenuation in female Wistar rats. The effect of SR on THC-induced antinociceptive was similar across an interval 35-120 minutes after the start of vapor inhalation in each group, suggesting a duration of action that extends from immediately post-inhalation for at least 90 minutes. This study also shows for the first time that the hypothermic response to e-cigarette vapor

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inhalation of THC is altered by prior treatment with the CB_1 inverse agonist/antagonist compounds SR or AM251. Interestingly, the effects of SR or AM251 on hypothermia manifested as a more rapid return to baseline body temperature following an apparently unaffected initial hypothermia observed in the first 60 minutes after the initiation of inhalation. This contrasted with the complete blockade of hypothermia induced by a 10 mg/kg, i.p. dose of THC that was observed when either SR or AM251 were administered prior to the THC injection, an observation that is consistent with prior results (Lichtman, Poklis, Poklis, Wilson & Martin, 2001; Marshell et al., 2014; McMahon & Koek, 2007; Son et al., 2010). There was no effect of the CB_2 receptor antagonist/inverse agonist compound AM630 on the hypothermic response to inhaled THC, confirming the expected selectivity for the CB_1 receptor.

It is uncertain at present why the two CB_1 antagonist/inverse agonist compounds did not block the initial hypothermia following inhalation. When administered in these doses, the hypothermia caused by 10 mg/kg THC, i.p., was completely blocked by SR (also see Nguyen et al., 2016) or AM251 as would be predicted from precedent literature. As one minor caveat, a similar delay in the ability of SR to counter the effects of injected THC have occasionally be reported (Fernandez-Ruiz, Munoz, Romero, Villanua, Makriyannis & Ramos, 1997; Taffe, Creehan & Vandewater, 2015). Critically, this phenomenon extended in the present study to male Sprague-Dawley rats which had not been exposed to repeated-inhalation conditions on sequential days (Study 5), which is an important generalization and replication of the effect observed in the female Wistar rats. This is not likely to be due to a significantly higher peak THC dose from inhalation compared with injection, as we previously showed equivalent peak plasma THC after 200 mg/mL inhalation (30 minutes) and 10 mg/kg, i.p. (Nguyen et al 2016). The nadir of the body temperature observed 60 minutes after the start of inhalation was similar to the nadir reached 60-90 minutes after i.p. injection which also suggests a similar THC dose. The difference in SR or AM251 effect across routes of THC administration may be due to the rapidity with which THC enters the brain, however this would require further investigation to confirm. Since effects of SR on tail-withdrawal were consistent for 90 minutes starting from immediately post-session, this diference with the thermoregulatory response may suggest differences in THC penetration of hypothalamic regions over spinal-medullary circuitry (Advokat & Burton, 1987; Fields, Bry, Hentall & Zorman, 1983; Fitton & Pertwee, 1982).

Overall this work further confirms the efficacy of a new electronic-cigarette based method of delivering THC to rats. Since humans are increasingly using e-cigarettes to use cannabis extracts, it is necessary to develop pre-clinical models for evaluation of the effects of THC (and other cannabinoids) with this route of administration. This study confirms first that tolerance to the hypothermic and antinociceptive effects of THC can be produced with this method. It also further confirms the role of CB1 receptors in mediating the effects of inhaled THC. Thus, this method offers excellent face and construct validity for the investigation of the consequences of vapor inhaled THC.

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Author Contributions:

MAT and JDN designed the studies, with refinements contributed by MC, KMC, YG and SAV. MC created the vapor inhalation equipment that was used. JDN, KMC, YG and SAV performed the research and conducted initial data analysis. JDN and MAT conducted statistical analysis of data, created figures and wrote the paper. All authors approved of the submitted version of the manuscript.

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Competing Interest: MC is proprieter of LJARI which markets vapor-inhalation equipment. SAV consults for LJARI.

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