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RESEARCH ARTICLE

Sex-specific impact of repeated adolescent vapour exposure to JWH-018 on dopamine response, behaviour and pharmacokinetics across adolescence and adulthood

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Abstract

Background and Purpose: Alarming trends show that vaping e-cigarettes containing synthetic cannabinoid receptor agonists, such as JWH-018, is increasing among youth. However, the effects of these trends are unclear in both sexes. We therefore characterized the neuropharmacological effects of adolescent JWH-018 inhalation in male and female rats.

Experimental Approach: Adolescent rats inhaled passively JWH-018 vapour (0.3 or 0.6 mg·ml^{-1} qd) for 21 days. During vapour exposure, JWH-018 and main metabolite plasma levels, body weight, locomotion and ultrasonic vocalizations were measured at different time points. During drug-free period, behavioural (withdrawal signs, anxiety and repetitive-like behaviour) and microdialysis (NAc shell/mPFC dopamine responsiveness to intraoral chocolate, taste reactivity) studies were performed 24 h and 7 days after last JWH-018 inhalation, respectively.

Key Results: Repeated adolescent JWH-018 inhalation induced sex-dependent effects with (i) higher plasma levels in males; (ii) increased body weight gain and withdrawal signs in females; (iii) transient hypolocomotion in females and dose-dependent biphasic locomotion in males; (iv) higher taste aversion in male; (v) sex- and dose-dependent adaptive changes of NAc shell and mPFC dopamine to single/repeated chocolate exposure in early adulthood, as follows: in the NAc shell, either low or high dose decreased dopamine sensitivity to chocolate in males, low dose abolished habituation whereas high dose blunted dopamine responsiveness in females; in the mPFC, the low dose blunted responsiveness in male and induced habituation in females while the high dose induced habituation only in males.

Abbreviations: BW, body weight; CB, cannabinoid; eCB, endocannabinoid; EPM, elevated plus maze; JWH-018, 1-pentyl-1H-indol-3-yl- (1-naphthalenyl)- methanone; JWH-018, 4-hydroxyindole, 4-hydroxy-1-pentyl-1H-indol-3-yl-(1-naphthalenyl)-methanone; JWH-018, 5-hydroxyindole, 5-hydroxy-1-pentyl-1H-indol-3-yl-(1-naphthalenyl)-methanone; MB, marble burying; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; SCRA, synthetic cannabinoid receptor agonist; TRT, Taste reactivity test; UHPLC, ultra-high performance liquid chromatography with tandem mass spectrometry; USVs, ultrasonic vocalizations; VTA, ventral tegmental area.

Conclusion and Implications: Using highly translational models, we showed that the impact of adolescent JWH-018 inhalation differs between sexes.

KEYWORDS
cannabinoids, chocolate, e-cigarette, microdialysis, sex differences

1 | INTRODUCTION

Synthetic cannabinoid receptor agonists (SCRAs) are a major class of novel psychoactive substances (NPSs) (EMCDDA, 2022; Miliano et al., 2016). The emergence of new NPSs has reshaped the public health landscape, due to their use by adolescents, often unaware of their pharmacological effects and safety profiles (Fattore & Fratta, 2011; Pintori et al., 2017). In Europe, more than 220 distinct SCRAs have been detected in herbal mixtures and e-cigarette solutions of increasing potency compared to the early SCRAs generation (e.g., naphthoylindoles such as **JWH-018**) (EMCDDA, 2022). The use of SCRAs is steadily increasing worldwide (EMCDDA, 2024), particularly among adolescents attracted by vaping SCRAs via e-cigarettes (Cozier et al., 2024; EMCDDA, 2022). Beyond intentional use, growing concerns arise from accidental intoxications in adolescents due to vaping liquids adulterated with SCRAs (Craft et al., 2024; Oomen et al., 2022; Slob et al., 2025).

Unlike **Δ9-tetrahydrocannabinol** (THC), a weak partial agonist of cannabinoid receptors (CB receptors), SCRAs are highly potent full agonists of both the **CB_1** receptor and **CB_2** receptor (De Luca et al., 2016; N. Pintori et al., 2017). Furthermore, Phase I metabolites of some SCRAs, such as monohydroxylated derivatives of JWH-018, have been shown to exhibit CB_1 receptor activity both in vitro and in vivo (Brents et al., 2011). Thus, SCRAs induce more severe withdrawal symptoms and cognitive impairment than THC, elevating the risks for cannabis use disorder and psychiatric comorbidities with prolonged use (Papanti et al., 2014; Schifano et al., 2015). In addition to their **dopamine** (DA) stimulant effects and reinforcing properties in rodents (De Luca et al., 2015; Margiani et al., 2022), recent studies have demonstrated that repeated low-dose JWH-018 exposure (0.25 $mg \cdot kg^{-1}$, intraperitoneal, 14 days) induces behavioural disturbances (e.g., anxiety-like behaviours, withdrawal signs) and disrupts mesocorticolimbic DA transmission, impairing DA responsiveness in the medial prefrontal cortex (mPFC) and nucleus accumbens (NAc) to motivational stimuli. These behavioural and neurochemical alterations are associated with neuroinflammation and CB_1 receptor downregulation (Pintori et al., 2021; Pintori, Mostallino, et al., 2024), underscoring the detrimental effects and possible psychiatric relevance of SCRA use.

Although smoking represents the predominant route of SCRA self-administration in humans, the majority of preclinical studies on these substances employ parenteral administration routes, with few exceptions (Lefever et al., 2017; Marshell et al., 2014; Wiebelhaus et al., 2012). Consequently, non-combustible delivery validated methods (e.g., e-cigarettes and vaporizers) to study cannabinoids by animal research have expanded in recent years, improving alignment with human consumption patterns (Moore et al., 2022). Nevertheless, despite rising adolescent SCRA vaping, the consequences of inhaling these substances during adolescence, as well as potential sex-dependent vulnerabilities, remain underexplored. To fill this gap, we investigated the neuropharmacological effects and plasma pharmacokinetic profiles of JWH-018 and two of its major hydroxylated metabolites (Brents et al., 2011) following recurrent inhalation during adolescence in rats. Considering the evidence of sex-dependent cannabinoid effects, particularly during neurodevelopment (Liana Fattore & Fratta, 2010; L. Fattore et al., 2007; Melis et al., 2013), we hypothesised that adolescent JWH-018 exposure would illicit sex-specific outcomes, providing insights into sex-dependent vulnerabilities and consequences of SCRAs exposure. To test this hypothesis, adolescent male and female Sprague–Dawley rats were passively exposed to JWH-018 vapour (0.3 or 0.6 $mg \cdot ml^{-1}$) once daily for 21 consecutive days (postnatal day 35–55). First, we characterized the pharmacokinetic profiles of JWH-018 and two monohydroxylated metabolites (JWH-018 4-hydroxyindole

What is already known?

- The inhalation of synthetic cannabinoid receptor agonists (SCRA) via e-cigarettes is rising among youths worldwide.
- Repeated JWH-018 exposure in adult rats induces behavioural, molecular and neurochemical dysregulations in mesocorticolimbic circuitry.

What does this study add?

- Sex and dose significantly affect the behavioural and neurochemical effects of JWH-018 inhalation during adolescence.
- Sex affects the pharmacokinetic profiles of JWH-018 and its metabolites after single and repeated inhalation.

What is the clinical significance?

- Our data confirm the risks associated with recurrent SCRA use during a critical neurodevelopmental period.
- JWH-018 inhalation may be associated with sex-dependent pharmaco-toxicological effects and health risks.

and 5-hydroxyindole) in the plasma of adolescent rats following both acute and repeated inhalation. Subsequently, at various time points during the inhalation, we assessed physiological (i.e., body weight) and behavioural (i.e., locomotor activity and ultrasonic vocalizations [USVs]) changes, as well as spontaneous somatic withdrawal signs, anxiety-like, affectivity and repetitive behaviours during the acute withdrawal phase. Finally, in early adulthood, we evaluated DA responsiveness in the NAc shell and mPFC, along with taste reactions to repeated exposure to a natural rewarding stimulus (i.e., intraoral chocolate), to determine the impact of adolescent JWH-018 inhalation on novelty and DA-related salience attribution of stimuli.

2 | METHODS

2.1 | Animals

Male and female Sprague–Dawley (SD) rats were used (Envigo, Italy). At arrival (postnatal day 21), animals were housed in groups of six in standard plastic cages with wood chip bedding, at temperature of 22°C ± 2°C and 60% humidity and under a 12-h light/dark cycle (lights on from 7:00 AM). Tap water and standard laboratory rodent chow (Mucedola, Settimo Milanese, Italy) were provided ad libitum in the home cage. All animal care and experimental procedures were carried out in accordance with European Council directives (609/86 and 63/2010) and in compliance with the animal policies issued by the Italian Ministry of Health and the Committee for Animal Wellbeing (OPBA, University of Cagliari). Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020). Authors state that they have complied with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis (Curtis et al., 2025).

2.2 | Experimental design timeline

Following the acclimation and handling period (postnatal day 21–34), adolescent male and female SD rats were passively exposed daily to JWH-018 (0.3 or 0.6 mg·ml^{-1}) or vehicle vapour using LJARI vapour chambers (La Jolla, USA) for 21 consecutive days (postnatal day 35–55) (Figure 1). The doses of JWH-018 were selected based on preliminary pilot experiments. Animals were randomly assigned to different experimental groups (G) for in vivo evaluations (see Tables S1 and S2). First, separate groups of male and female adolescent rats (G1) were used to characterize the pharmacokinetic profile of JWH-018 and its two first-pass active monohydroxylated metabolites, JWH-018 4-hydroxyindole [4-hydroxy-1-pentyl-1H-indol-3-yl-(1-naphthalenyl)-methanone] and JWH-018 5-hydroxyindole [5-hydroxy-1-pentyl-1H-indol-3-yl-(1-naphthalenyl)-methanone], in plasma following either acute or repeated inhalation. Subsequently, at various time points during the vaping period, body weight gain and locomotor activity were assessed in different groups of rats (G2) to evaluate potential alterations in those parameters related to JWH-018 inhalation. Moreover, the emission of USVs, a behavioural marker of affective state in rats (Premoli et al., 2023), was evaluated at various time points during the vaping period. Based on our previous study, 24 h after the final JWH-018 vaping session, spontaneous somatic withdrawal signs, anxiety-like behaviours (assessed using the Elevated Plus Maze, EPM) and repetitive-like behaviours (evaluated via the Marble Burying test, MB) were measured to determine the presence of acute withdrawal symptoms and affective state disturbances. In early adulthood (postnatal day 62), different groups of rats (G3) were repeatedly exposed to a natural rewarding stimulus (i.e., intraoral chocolate). Taste reactivity tests (TRTs) and in vivo microdialysis were conducted to investigate whether JWH-018 inhalation during adolescence induces persistent behavioural alterations and dysregulations in dopamine (DA) responsiveness in the medial prefrontal cortex (mPFC) and

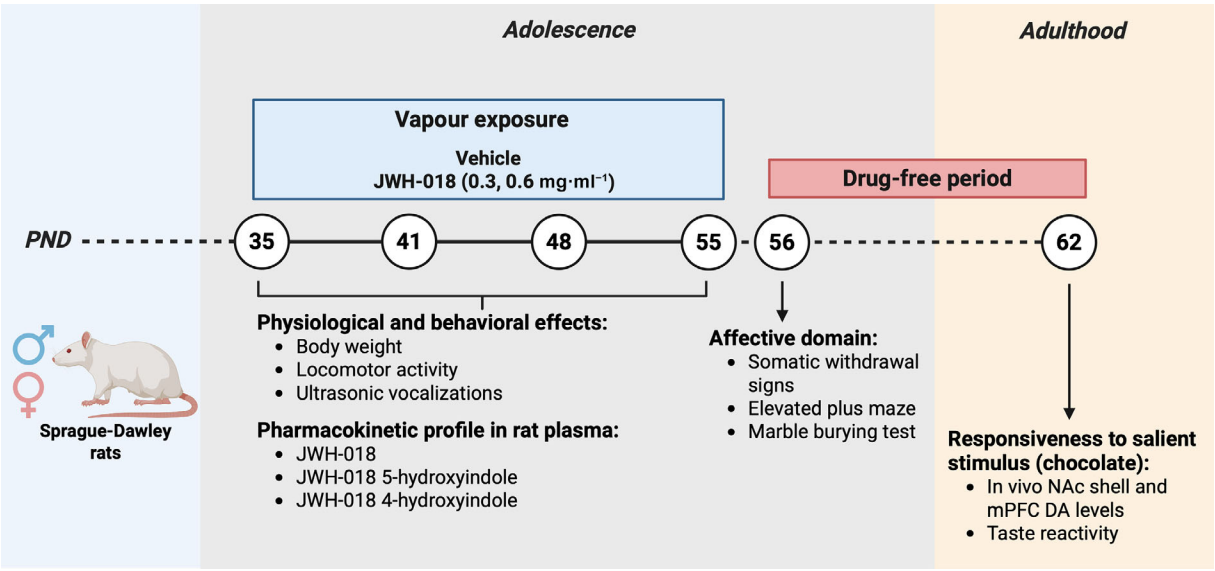

FIGURE 1 Experimental design timeline. DA, dopamine; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; PND, postnatal day.

nucleus accumbens (NAc) shell, as previously observed in adult rats (Pintori et al., 2021). The sample sizes for each experimental group (n ≥ 5) were determined based on power analysis calculations derived from the observed variability in our prior published studies (Pintori et al., 2021; Pintori, Mostallino, et al., 2024; Pintori, Serra, et al., 2024). The specific groups and the number of animals used in each test are detailed in Tables S1 and S2, and shown on Figure legends. Due to technical issues (e.g., catheter or dialysis probe obstruction, sample preparation errors), some animals or samples were excluded from statistical analysis, resulting in reduced group sizes in a few cases. Data evaluation and analysis were performed by blinded experimenters.

2.3 | Vapour exposure

2.3.1 | Vapour exposure equipment

The vapour chamber used in this project was designed and manufactured by La Jolla Alcohol Research Inc. (LJARI; San Diego, CA) and was controlled by FSR LJARI Single Chamber E-Vape™ Control System. LJARI vapour generator was fourth generation, model 0004–100 W, which rapidly heated the stainless-steel coil in the tanks at 61.1 W, 0.4 Ω, to 232.2°C during the 5-s puff deliveries. The chamber consisted of a 58.5 × 48.8 × 26.7 cm clear, air-tight acrylic box, capable of holding two 21.0 × 47.4 × 21.0 cm clear plastic tub cages with wire tops. One port delivered vapour integrated with ambient air into the chamber at the upper level, and four outlet ports allowed a vacuum pump (1.42 psi air compressor) to pull air and aerosol out of the chamber at a steady rate of 1 L·min^{-1} (achieved via a regulator and flow gauge), resulting in clearing of vapour from the chamber approximately 2 min after completion of a puff. The exhaust was filtered through a Whatman HEPA-CAP filter and routed to a fume hood for safe clearance.

2.3.2 | Vapour exposure procedure

The protocol was based and adapted from vaping THC literature (Nguyen et al., 2020; Ruiz et al., 2021). Briefly, six rats were placed in groups of three into tub cages with bedding and then received vaporized solutions of JWH-018 (0.3 or 0.6 mg·ml^{-1}) or vehicle. Each vaping session lasted 30 min, and 14 puffs of 5 s each were delivered every 120 s. Approximately 1 ml of solution was vaporized in the 30-min session (last puff at 28 min). During vaping period, rats were daily weighed and monitored in order to evaluate animal welfare and/or any signs of distress.

2.4 | Pharmacokinetic analyses

2.4.1 | In vivo procedures

Separate groups of male and female rats were used for pharmacokinetic studies following single and repeated JWH-018 inhalation. Rats were anaesthetised with isoflurane and implanted with an intrajugular vein catheter made of polyethylene (PE) tubing (Portex Ltd, Hythe, England) (ID 0.58 mm, OD 0.96 mm) 24 h before the pharmacokinetic experiments. Based on our previous studies (De Luca et al., 2022; Mostallino et al., 2025; Piras et al., 2024), this short recovery period (24 h) is enough to obtain the full recovery of animal welfare after a 15-min surgery session. The next day, a sample of blood was collected 30 min before the start of vaping session (i.e., blank sample). For the pharmacokinetic assessment after repeated inhalation, the blank sample was collected ∼48 h after the 20th JWH-018 vaping session. Then, animals were removed from the vapour chamber immediately after the 30-min session (∼2 min after the last puff), individually placed into cages with bedding, and blood samples were collected at 5, 15, 30, 60 and 120 min after the end of the vaping session. The 200 µl of blood was collected from the jugular catheter and centrifuged for 15 min at 34878 × *g* to extract the plasma, which was stored at −80°C until analysis.

2.4.2 | Chemical/reagents

Analytical LC-grade methanol, acetonitrile, formic acid and ammonium acetate were purchased from Sigma Aldrich (Milan, Italy). Bi-distilled water, (<18 MΩ cm^{-1} at 25°C) was obtained with a MilliQ purification system (Millipore, Milan, Italy) and used for all preparations. JWH-018 pure standard, JWH 018 d11, JWH 018 5-hydroxyindole, JWH 018 5-hydroxyindole d5, JWH 018 4-hydroxyindole and JWH 018 4-hydroxyindole d9 were purchased from LGC Standards (Milan, Italy).

2.4.3 | Sample preparation for UHPLC-IM-QTOF-MS analysis

The protocol for sample preparation was adapted from Kronstrand et al. (2014). Before analysis, plasma samples were subjected to extraction adding 50 µl of plasma and 200 µl of 0.4-M ammonium acetate aqueous solution. Samples were vortexed and placed in an oven at 55°C for 1 h. Thereafter, 400 µl of cold acetonitrile were added. The solution obtained was centrifuged at 34878 × *g* for 10 min, and 500 µl of the supernatant were transferred into a glass vial and dried under a gentle nitrogen stream. The dried phase was reconstituted with 100 µl of acetonitrile:methanol:water mixture (6/6/4, *v/v*). All samples were injected in UHPLC–MS/MS and acquired in positive ionization mode.

2.4.4 | Calibration standards

Calibration standards were prepared by spiking 1 ml of drug-free plasma with diluted methanolic JWH-018 standard solutions. The final concentrations of the calibrators were 0.01, 0.1, 1, 5 and 10 ng·ml^{-1}. Calibration standards were processed as described above.

2.4.5 | UHPLC–MS/MS analysis of JWH-018 levels

The protocol was adapted from Toennes et al. (2017). Reconstituted samples were analysed with an Agilent 1290 Infinity II LC coupled via JetStream electrospray interface (ESI) with an Agilent 6470 Triple Quad mass spectrometer. An aliquot of 3.0 µl from each sample was injected in a Kinetex 5 µm EVO C18 100 A, 150 mm × 2.1 µm column (Agilent Technologies, Palo Alto, CA). The column was maintained at 50°C at a flow rate of 0.4 ml·min^{-1}. The mobile phase consisted of (A) water containing 0.01% formic acid and (B) acetonitrile containing 0.01% formic acid. The chromatographic separation was obtained with the following gradient: initially 80% of A, then a linear decrease from 45% to 35% of A in 4 min, then at 30% in 2 min. Subsequently, the mobile Phase A was again decreased from 30% to 15% in 2 min and stayed at this percentage for 1 min, and then brought back to the initial conditions in 2 min. The source was operated in positive ion mode with the following parameters: gas temperature, 300°C; gas flow (nitrogen) 5 L·min^{-1}; nebulizer gas (nitrogen), 30 psi; sheath gas temperature, 250°C; sheath gas flow, 9 L·min^{-1}; capillary voltage 3500 V; nozzle voltage 500 V; fragmentor 150 V; skimmer 65 V, octapole RF 7550 V; capillary voltage, 3.5 kV; collision energy 20 eV, mass precursor per cycle = 3. High-purity nitrogen (99.999%) was used as a drift gas with a trap fill time and a trap release time of 2000 and 500 µs, respectively. The MS/MS was operated in multiple reaction monitoring mode (MRM) with two transitions recorded for the analyte. In Time Segment 1 (start time 2.2 min), the transitions were 342.2 → 155 and 342.2 → 127. The Agilent MassHunter LC/MS Acquisition was used for data acquisition.

2.4.6 | Method validation

Under our experimental conditions, a standard mixture containing JWH-018, JWH-018 4-hydroxyindole and JWH-018 5-hydroxyindole was injected at different concentrations to determine the limit of detection (LOD) and limit of quantitation (LOQ) values.

The sensitivity of the method was calculated based on the signal-to-noise ratio, which is 3:1 and 10:1 (LOD and LOQ), respectively. The values obtained from LOD and LOQ are reported in Table S3.

The repeatability of the method was evaluated by injecting the calibrant mixture six times for each concentration. The relative standard deviation (RSD) of JWH and its metabolites was found to be between 0.9% and 2.8%. The intermediate precision was evaluated by data generated on two different days. By injecting the same standard mixture, the difference in the mean from 2 days ranged from 0.01% to 2.59%.

2.5 | Evaluation of body weight

Body weight (BW) was monitored throughout the vaping period (postnatal day 35–55). The BW increments (Δ) in grams per animal at each time point (i.e., vaping session) were calculated as the difference between ΔBW at a given day (Day *t*) and BW at Day 1, that is before the first vaping session (ΔBW = BW-t − BW Day 1).

2.6 | Behavioural tests

To prevent any confounding effects from environmental or conditioned stimuli, all behavioural assessments were carried out in dedicated experimental rooms, distinct from the vaporization room.

2.6.1 | Locomotor activity

Locomotor activity was recorded from individual rats placed in Plexiglas cages (*L* 47 cm × *H* 19 cm × *W* 27 cm) equipped with infrared photocell emitters and detectors situated along their long axis (Opto-Varimex, Columbus Instruments, Columbus, OH, USA). Locomotor activity, which consisted of locomotion along the axes of the cage, was scored by a counter that recorded the number of interruptions of each infrared beam and the total number of beam interruptions. Locomotor activity was recorded before and immediately after the 1st, 7th, 14th and 21st vapour exposure session at 15-min interval for a total of 30 min (pre-vaping) and 120 min (post-vaping), respectively.

2.6.2 | Emission of ultrasonic vocalizations (USVs)

Rats were individually placed in Plexiglas cylinders (diameter, 30 cm; height, 30 cm) with the bottom covered with fresh bedding. Cylinders had one half totally painted black and the other half partially painted black to form alternating horizontal black and transparent stripes and were topped with a lid equipped with an ultrasonic microphone (CM16/CMPA, Avisoft, Berlin, Germany). The microphone was placed at an average distance of 25 cm from rats and was connected to an ultrasound-recording device (UltraSoundGate 116 Hb, Avisoft, Berlin, Germany); constant gain was maintained throughout recordings. The emission of USVs was recorded in two separate sessions of 15 min (pre-vaping) and 30 min (post-vaping) on the 1st, 7th, 14th and 21st vapour exposure session. The software SASLab Pro 4.52 (Avisoft, Berlin, Germany) was used to convert USV recordings into spectrograms with the following settings: 512 FFT-length, Hamming window and 75% overlap frame set-up (Simola et al., 2012). Afterwards, spectrograms were inspected by an experimenter blind to treatments who cleaned all signals that could not be unambiguously classified as USVs. The SASLab Pro 4.52 software was then used to count the numbers of 22-kHz USVs (aversive) and 50-kHz USVs (appetitive), defined according to the criteria previously described (Simola & Brudzynski, 2018). The total numbers of 22- and 50-kHz USVs were scored (Burgdorf et al., 2008). Moreover, the USVs of 22-kHz were used as an index of aversive state (Brudzynski, 2013), potentially associated with withdrawal state (Covington & Miczek, 2003) (pre-inhalation testing) and/or vapour exposure procedure (post-inhalation testing).

2.6.3 | Spontaneous somatic signs of withdrawal

Rats were individually placed in plastic cages (*L* 30 × *H* 25 × *W* 45 cm) with standard rat bedding. Cages were located in a sound-proof room for behavioural observation. Point scoring was performed by an observer (placed behind a one-way window), blind to the treatment. Based on previous studies (Aceto et al., 2001; Diana et al., 1998), spontaneous signs of cannabinoid withdrawal were scored by counting the total number of events such as scratching, wet dog shakes, facial rubbing and licking, over a 30-min test period (Diana et al., 1998; Pintori et al., 2021). This specific timepoints was chosen based on our previous study (Pintori et al., 2021) showing spontaneous somatic withdrawal signs 24 h but not 7 days—except for biting—after repeated JWH-018 exposure (0.25 mg·kg^{-1}, intraperitoneally, i.p., 14 consecutive days).

2.6.4 | Elevated plus maze

The elevated plus maze (EPM) is used to evaluate spatial anxiety in rodents (Morales et al., 2010). The EPM was made of white PVC and consisted of two opposite open arms (*L* 50 × *W* 10 cm) and two opposite closed arms (*L* 50 × *W* 10 cm), the latter enclosed by 40 cm high walls along their length. The four arms converged to a central square (*L* 10 × *W* 10 cm), thus reproducing the shape of a plus sign. The apparatus was elevated 50 cm from the floor. Rats having no prior experience of the EPM were placed in the central square and left free to explore the whole apparatus for a single 5-min test session. The experiments were performed under a uniform illumination of 40 lx. Rats' performance was videotaped and later evaluated by an experimenter blind to treatments to calculate the percentages of arm entries and of the time spent in the open and closed arms with respect to the total number of arm entries and the total amount of time spent in the arms, respectively. A rat was considered inside a specific arm when it had all four paws inside that arm.

2.6.5 | Marble burying test

The marble burying (MB) is used to evaluate compulsive-like activity/repetitive behaviours in rodents (Zanda et al., 2017). The MB was conducted in an open transparent plastic cage (*L* 34.5 × *H* 20 × *W* 54 cm) with 5 cm of fresh hardwood chip bedding. Twenty-four standard glass marbles (1.5 cm in diameter, arranged in six rows of four marbles each) were placed uniformly over the bedding surface. Individual rats were placed in the test cage, and the activity was monitored for 30 min by a video camera placed above the cage. At the end of the session, animals were gently removed from the cages, and the number of marbles partially (≥67%) and totally (>95%) buried were counted as previously described (Pintori et al., 2021; Zanda et al., 2017). New bedding was used for each animal.

2.6.6 | Taste reactivity test (TRT)

The TRT represents a sensitive technique offering a detailed window into the intrinsic hedonic and aversive evaluations of gustatory stimuli in rodents (Grill & Norgren, 1978). The unique methodological approach of the TRT, which involves direct intraoral infusion, along with the assessment of aversive reactions, provides a direct index of palatability that can be used to assess taste neophobia, as previously observed after repeated exposure to drug of abuse, such as SCRAs (Pintori et al., 2021). During the 5-min intraoral chocolate infusion, either naive or pre-exposed animals were monitored, and two classes of taste reactivity patterns were scored, that is, positive hedonic (appetitive) and negative hedonic (aversive). Positive hedonic reactions were paw licks, lateral tongue protrusions and rhythmic tongue protrusion; aversive reactions were face washing, forelimb flails, gapes, chin rubs, paw tread and locomotion (De Luca et al., 2012). Each reaction was scored and assigned one point if it lasted 1–5 s and two points if it lasted more than 5 s.

2.7 | Chocolate exposure

2.7.1 | Preparation of oral catheters

The oral catheters were made of a 22 G stainless steel needle and polyethylene (PE) tubing (Portex Ltd, Hythe, England) (ID 0.58 mm, OD 0.96 mm) as previously described (Pintori et al., 2021). The needle was cut at one side (total length of 2 cm from the tip); the cut part was blunted and inserted in the PE tubing, which ended with a perforated circular disc.

2.7.2 | Surgery

In the same surgery session for the insertion of microdialysis probes, an oral catheter was inserted at the level of the first molar, passed along the space between the temporalis muscle and the skull by the tip of the 22-G needle and fixed on the top of the rat's head with a small plastic tip filled with cyanoacrylate glue.

2.7.3 | Infusion of chocolate solution

The oral catheter was connected to an infusion pump and the chocolate solution was pumped at a constant rate of 0.2 ml·min^{-1}, to a total of 1 ml per 5 min for each chocolate exposure (Pintori et al., 2021). The time interval between the two chocolate exposures was 4 h.

2.8 | In vivo microdialysis in freely moving animals

2.8.1 | Preparation of microdialysis probes

Vertical microdialysis probes, with an active dialysing portion of 1.5 mm for the NAc and 3 mm for the mPFC, were prepared with

AN69 fibres (Hospal Dasco, Italy) as previously described (De Luca et al., 2015).

2.8.2 | Surgery

At early adulthood (postnatal day 61), rats were anaesthetized with isoflurane and implanted with vertical dialysis probes in the NAc shell (A: +2.2, L: +1.0 from bregma, V: 7.8 from dura) or in the mPFC (A: +3.7, L: +0.8 from bregma, V: 5.0 from dura), according to the Rat Brain Atlas coordinates (Paxinos & Watson, 2007).

2.8.3 | Dopamine assessment

One day after surgery, probes were perfused with Ringer's solution (composition in mM: 147 NaCl, 4 KCl, 2.2 $CaCl_2$) at a constant rate of 1 µl·min^{-1}. Dialysate samples (10 µl) were injected into an HPLC equipped with a reverse phase column (C8 3.5 µm, Waters, USA) and a coulometric detector (ESA, Coulochem II) to quantify DA. The sensitivity of the assay for DA is 5 fmoles per sample. After 2-h washing, basal DA levels were evaluated and estimated as the mean of three consecutive samples whose values did not differ more than 10%. Then, rats were exposed two times to a salient taste stimulus (chocolate solution, 1 ml/5 min, see above) and DA levels were assessed for the next 2 h.

2.8.4 | Histology

At the end of the microdialysis experiment, animals were sacrificed with an overdose of isoflurane and their brains removed and stored in formalin (8%) for histological examination to verify the correct placement of the microdialysis probe.

2.9 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2025). All animals tested were treated as independent values, and no technical replicates were included in the analysis. Numerical data are presented as individual values or mean ± standard error of the mean (SEM) for parametric analyses or as median with 95% confidence intervals (CI) for non-parametric analyses. Data normality was assessed using the Shapiro–Wilk test. When data were found not to be normally distributed, non-parametric tests (e.g., Kolmogorov–Smirnov test, Welch's *t* test) were employed. To analyse sex differences (using a two-way ANOVA with sex and treatment as factors), all treatment groups (i.e., Veh, JWH-018 0.3 mg·ml^{-1} and JWH-018 0.6 mg·ml^{-1}) were included in each comparison, except for pharmacokinetics studies.

For body weight analysis, the effect of treatment (i.e., Veh, JWH-018 0.3 mg·ml^{-1}, JWH-018 0.6 mg·ml^{-1}) within each sex was evaluated using repeated measures (RM) two-way ANOVA (treatment × session), followed by Tukey's post hoc multiple comparisons test. Additionally, to assess the effects of sex, an overall analysis of changes in body weight (ΔBW) during the vaping period was conducted by calculating the area under the curve (AUC). The AUC was derived by plotting ΔBW values against the day of vaping and applying the trapezoidal rule. The resulting AUC values were compared using two-way ANOVA (sex × treatment), followed by Sidak's multiple comparisons test.

For locomotor activity, the effects of sex within each vaping session (pre- and post-inhalation testing periods) on total locomotion were analysed using two-way ANOVA (sex × treatment), followed by Sidak's multiple comparisons test. Repeated measures (RM) two-way ANOVA followed by Tukey's multiple comparisons test was used to evaluate the effects of treatment within sexes on locomotor activity within and between vaping sessions (treatment × time, treatment × session), and on DA responses in the NAc shell and mPFC following each chocolate exposure (treatment × time). The same statistical approach (RM Two-way ANOVA, treatment × session) was used to assess the effects of treatment on emissions of either 22- or 50-kHz USVs between vaping sessions (pre- and post-inhalation testing periods) within each sex. For RM tests, whenever we could not assume sphericity, a Geisser–Greenhouse correction was carried out by GraphPad Prism 8 software (GraphPad Prism, RRID:SCR_002798).

Potential pre-existing group differences in DA levels between sexes before each chocolate exposure were analysed using two-way ANOVA (sex × treatment), followed by Sidak's multiple comparisons test, while the effects of treatment within sexes before each chocolate exposure were analysed using one-way ANOVA, followed by Tukey's multiple comparisons test. The same statistical approaches were used to assess the effects of sex (two-way ANOVA, sex × treatment) and treatment (one-way ANOVA) on behavioural assessments (EPM, MB test, spontaneous withdrawal signs and TRT). Moreover, potential differences between hedonic and aversive reactions after each chocolate exposure within treatment and sex were analysed using two-way ANOVA (treatment × reaction), followed by Sidak's multiple comparisons test. Post hoc tests were conducted only if the F-value in the ANOVA reached a significance level of $P < 0.05$ and there was no significant variance in homogeneity. For pharmacokinetics studies, differences in each parameter (i.e., CMax, AUC) within treatment (JWH-018 0.3 mg·ml^{-1}, JWH-018 0.6 mg·ml^{-1}) between sexes were analysed using two-stage step-up (Benjamini, Krieger and Yekutieli) multiple unpaired t-tests. The same statistical approach was used to assess differences between single and repeated JWH-018 inhalation. Differences in plasma levels before the 21st vaping session (−30 min, see Figure 2) within treatment between sexes were analysed using an unpaired t-test. The effects of sex on time course for each analyte after single and repeated inhalation of either JWH-018 0.3 or 0.6 mg·ml^{-1} were analysed using RM Two-way ANOVA (sex x time), followed by Sidak's multiple comparisons

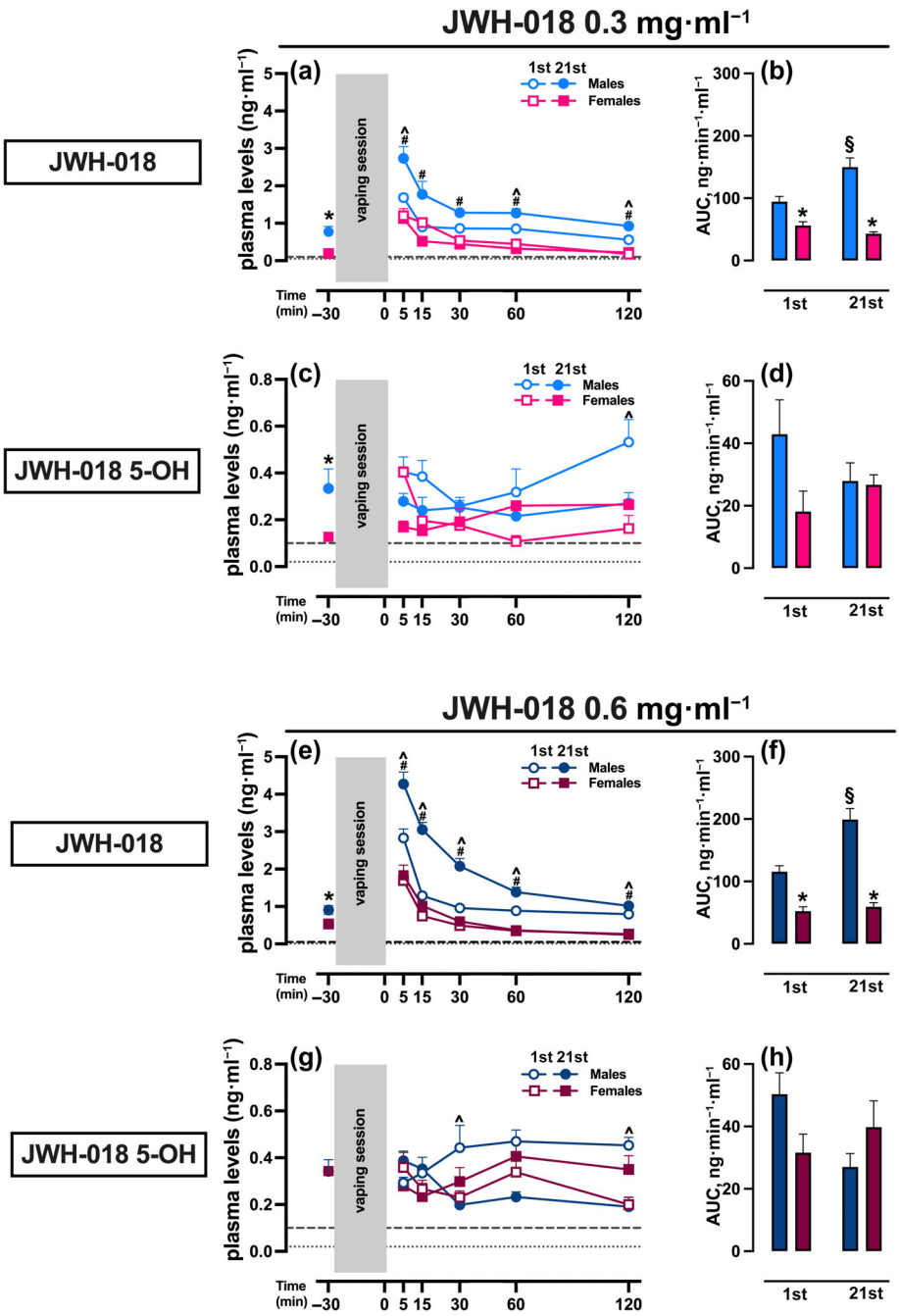

FIGURE 2 Plasma concentrations of JWH-018 and JWH-018 5-hydroxyindole in adolescent male and female rats after single and repeated inhalation. Data are presented as mean ± SEM of the time course, expressed as ng·ml^{-1}, of the JWH-018 (a, e) and JWH-018 5-hydroxyindole (c, g) concentration in plasma of adolescent male (light and dark blue circles) and female (light and dark pink squares) rats after single (i.e., first session, empty symbol) and repeated (i.e., 21st session, solid symbol) 0.3 or 0.6-mg·ml^{-1} JWH-018 inhalation (30-min vaping session). Dashed line represents the limit of quantification (LOQ), and dotted line represents the limit of detection (LOD) for each analyte. Please note that the single value at −30 min (a, c, e, g) refers only to repeated inhalation and that different scale bars for each analyte are used for clarity of presentation. * $P < 0.05$ males versus females (unpaired *t* test); ^ $P < 0.05$ males versus females after single inhalation; # $P < 0.05$ males versus females after repeated inhalation (RM two-way ANOVA). The bars represent total exposure, as measured by areas under the curves (AUC, nmol·min^{-1}·ml^{-1}) ± SEM, to JWH-018 (b, f) and JWH-018 5-hydroxyindole (d, h) in adolescent male and female rats after single (i.e., first session) and repeated (i.e., 21st session) 0.3- or 0.6-mg·ml^{-1} JWH-018 inhalation. Multiple unpaired *t* test. * $P < 0.05$ males versus females within treatment; § $P < 0.05$ single versus repeated inhalation within sex. Males: JWH-018 0.3 mg·ml^{-1} n = 6, JWH-018 0.6 mg·ml^{-1} n = 7; females: n = 8 per group. Repeated inhalation: males: JWH-018 0.3 mg·ml^{-1} n = 7, JWH-018 0.6 mg·ml^{-1} n = 8; females: JWH-018 0.3 mg·ml^{-1} n = 8, JWH-018 0.6 mg·ml^{-1} n = 7.

test. In this study, P values < .05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism 8 software (GraphPad Prism, RRID:SCR_002798).

2.10 | Drugs and solutions

JWH-018 was purchased from Tocris (Bristol, UK) and solubilized in 3.6% ethanol absolute, 2.4% tween-80, 94% vegetal glycerol (VG) and propylene glycol (PG) in a 1:1 ratio, which was also used the vehicle solution. A solution containing chocolate syrup (Nesquik Squeeze©, Nestle, Switzerland) and tap water (1:1) was used as a gustatory taste stimulus.

2.11 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in https://www.guidetopharmacology.org/ and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2023).

3 | RESULTS

3.1 | Pharmacokinetics profiles of JWH-018 and its monohydroxylated metabolites in adolescent rat plasma after single and repeated inhalation

Figures 2 and S5 show the time-course of JWH-018 and its first-pass metabolites, JWH-018 5-hydroxyindole and JWH-018 4-hydroxyindole, concentrations in adolescent male and female rats after single and repeated (21 sessions) 30-min exposure to 0.3 and 0.6 mg·ml^{-1} JWH-018 vapour. Table 1 reports peak concentration values (Cmax) in plasma, time at which Cmax was attained (Tmax), half-life ($t_{1/2}$) of elimination of JWH-018 and its 5-hydroxyindole metabolite and the area under the curve (AUC) after single and repeated inhalation of JWH-018 in male and female rats. After a single vaping session of each dose, the plasma pharmacokinetic profile of JWH-018 was similar in adolescent males and females, with Tmax reached after 5 min in each sex. However, AUC and Cmax values of JWH-018 were significantly higher or trended to be higher in males than females (0.3 mg·ml^{-1}: AUC, $t_{(12)} = 4.78$, $P < 0.01$, Cmax, $t_{(12)} = 2.07$, $P = 0.060$; 0.6 mg·ml^{-1}: AUC, $t_{(13)} = 5.50$, $P < 0.001$, Cmax, $t_{(13)} = 4.05$, $P < 0.010$; Figure 2a,b,e,f). Consistently, greater JWH-018 plasma levels were observed over time in males than females after single inhalation of 0.3 mg·ml^{-1} (5′, 60′ and 120′) and 0.6 mg·ml^{-1} (5′–120′) JWH-018 inhalation (RM two-way ANOVA: sex × time: 0. 3 mg·ml^{-1}: $F_{(4,48)} = 3.59$, $P < 0.05$; 0.6 mg·ml^{-1}: $F_{(4,52)} = 3.04$, $P < 0.05$; Figure 2a,e).

Differences between sexes in the pharmacokinetic profiles of JWH-018 5-hydroxyindole tended to be significant. For example, at both doses, the AUC was respectively 2.3 and 1.6 higher in males than females (0.3 mg·ml^{-1}: $t_{(12)} = 2.05$, $P = 0.063$; 0.6 mg·ml^{-1}: $t_{(13)} = 2.08$, $P = 0.058$; Figure 2f,h), with the max peak concentration reached (Tmax) later in males than females—120 min (0.3 mg·ml^{-1}) and 60 min (0.6 mg·ml^{-1}) in males versus 5 min in females (Table 1). Moreover, greater plasma levels of the 5-hydroxyindole metabolite were observed in males than in females at 30 and 120 min after single inhalation of 0.3 (120′) and 0.6 mg·ml^{-1} (30′ and 120′) doses (RM two-way ANOVA: sex × time: 0.3 mg·ml^{-1}: $F_{(4,48)} = 2.71$, $P < 0.05$; 0.6 mg·ml^{-1}: $F_{(4,52)} = 3.97$, $P < 0.01$; Figure 2c,g).

TABLE 1 Plasma Cmax, Tmax, $t_{1/2}$ and area under the curve (AUC) for JWH-018 and its first-pass metabolite (JWH-018 5-OH) in adolescent male and female rats after single or repeated inhalation (21 sessions) of JWH-018 (0.3 or 0.6 mg·ml^{-1}).

			Males				Females			
Vaping session	Exposure JWH-018 (mg·ml^{-1})	Analyte	Cmax (ng·ml^{-1})	Tmax (min)	$t_{1/2}$ (min)	AUC (ng·min^{-1}·ml^{-1})	Cmax (ng·ml^{-1})	Tmax (min)	$t_{1/2}$ (min)	AUC (ng·min^{-1}·ml^{-1})
1st	0.3	JWH-018	1.685	5	12.17	94.3*	1.201	5	25.28	56.13
		JWH-018 5-OH	0.531	120	-	42.93	0.403	5	12.14	18.15
	0.6	JWH-018	2.83*	5	11.6	115.6*	1.694	5	12.54	52.43
		JWH-018 5-OH	0.47	60	-	50.34	0.35	5	43.43	31.57
21st	0.3	JWH-018	2.734*	5	14.41	149.9*,**	1.128	5	12.45	43.03
		JWH-018 5-OH	0.278	5	28.97	27.93	0.265	120	-	26.72
	0.6	JWH-018	4.27*	5	21.27	199.3*,**	1.83	5	14.7	59.23
		JWH-018 5-OH	0.38	5	21.19	26.99	0.405	60	-	39.8

Note: Multiple unpaired *t* test. First vaping session: males: JWH-018 0.3 mg·ml^{-1} n = 6, JWH-018 0.6 mg·ml^{-1} n = 7; females: n = 8 per group; 21st vaping session: males: JWH-018 0.3 mg·ml^{-1} n = 7, JWH-018 0.6 mg·ml^{-1} n = 8; females: JWH-018 0.3 mg·ml^{-1} n = 8, JWH-018 0.6 mg·ml^{-1} n = 7. Bold values highlight statistically significant differences.

*P < 0.05 males versus females.**P < 0.5 1st versus 21st vaping session within sex.

Interestingly, repeated JWH-018 inhalation induced sex-specific plasma accumulation of JWH-018 and its 5-hydroxyindole metabolite. At both doses, males showed higher JWH-018 levels than females before the 21st vaping session, that is, 48 h after the 20th vaping session (0.3 mg·ml^{-1}: $t_{(6,12)} = 4.04$, $P < 0.01$; 0.6 mg·ml^{-1}: $t_{(13)} = 2.71$, $P < 0.05$, Figure 2a,e). Moreover, greater JWH-018 5-hydroxyindole levels were observed in males than females before the last 0.3 mg·ml^{-1} inhalation ($t_{[6,23]} = 2.44$, $P < 0.05$; Figure 2c). After repeated inhalation, the plasma pharmacokinetic profile of JWH-018 was similar in adolescent males and females, with the Tmax reached after 5 min in both sexes. Similarly to single inhalation, AUC, Cmax and plasma levels over time for JWH-018 were significantly greater in males than females after repeated inhalation of each dose (0.3 mg·ml^{-1}: AUC, $t_{(13)} = 7.63$, $P < 0.0001$; Cmax, $t_{(12)} = 5.22$, $P < 0.001$; plasma levels, $F_{(4,52)} = 2.57$, $P < 0.05$; 0.6 mg·ml^{-1}: AUC, $t_{(13)} = 0.63$, $P < 0.0001$; Cmax, $t_{(13)} = 7.09$, $P < 0.0001$; plasma levels, $F_{(4,52)} = 7.65$, $P < 0.0001$; Figure 2a,b,e,f). Noteworthy, male AUC values were also greater than those observed after the first vaping session (0.3 mg·ml^{-1}: $t_{(11)} = 3.13$, $P < 0.05$; 0.6 mg·ml^{-1}: $t_{(13)} = 4.04$, $P < 0.01$; Figures 2b,f). Sex-specific changes in Tmax for JWH-018 5-hydroxyindole metabolite were observed compared to the single inhalation profile. In particular, marked and opposite Tmax switching were observed with both JWH-018 doses, that is, a decrease in males (0.3 mg·ml^{-1}: from 120 to 5 min; 0.6 mg·ml^{-1}: from 60 to 5 min) and an increase in females (0.3 mg·ml^{-1}: from 5 to 120 min; 0.6 mg·ml^{-1}: from 5 to 60 min) (Table 1). Sex-specific changes in Tmax for JWH-018 5-hydroxyindole metabolite were observed compared to single inhalation profile. JWH-018 4-hydroxyindole values were <LOD after either single or repeated inhalation to each JWH-018 dose (Figure S5).

3.2 | Physiological and behavioural effects during JWH-018 repeated inhalation

Repeated JWH-018 inhalation during adolescence exerted sex-dependent effects on body weight gain and locomotor activity.

3.2.1 | Body weight

As shown in Figure 3a, adolescent JWH-018 inhalation altered body weight gain in female but not male rats. In females, two-way ANOVA of body weight gained over the vaping period showed a main effect of session [$F_{(10,630)} = 3198$, $P < 0.0001$], treatment [$F_{(2,63)} = 4.46$, $P < 0.05$] and a session × treatment interaction [$F_{(20,630)} = 2.75$, $P < 0.0001$]. Tukey's post hoc test revealed increased body weight gain in JWH-018 0.3 and 0.6 mg·ml^{-1} groups as compared to the Vehicle group from Day 11 of vaping up to the treatment cessation (Figure 3a). No differences in body weight gain among groups were observed in male rats (Figure 3a). Notably, differences in cumulative ΔBW (expressed as area under the curve) between sexes were observed, with males gaining more weight than females (two-way ANOVA: sex main effect, $F_{[1,118]} = 763.4$, $P < 0.0001$; Figure 3b).

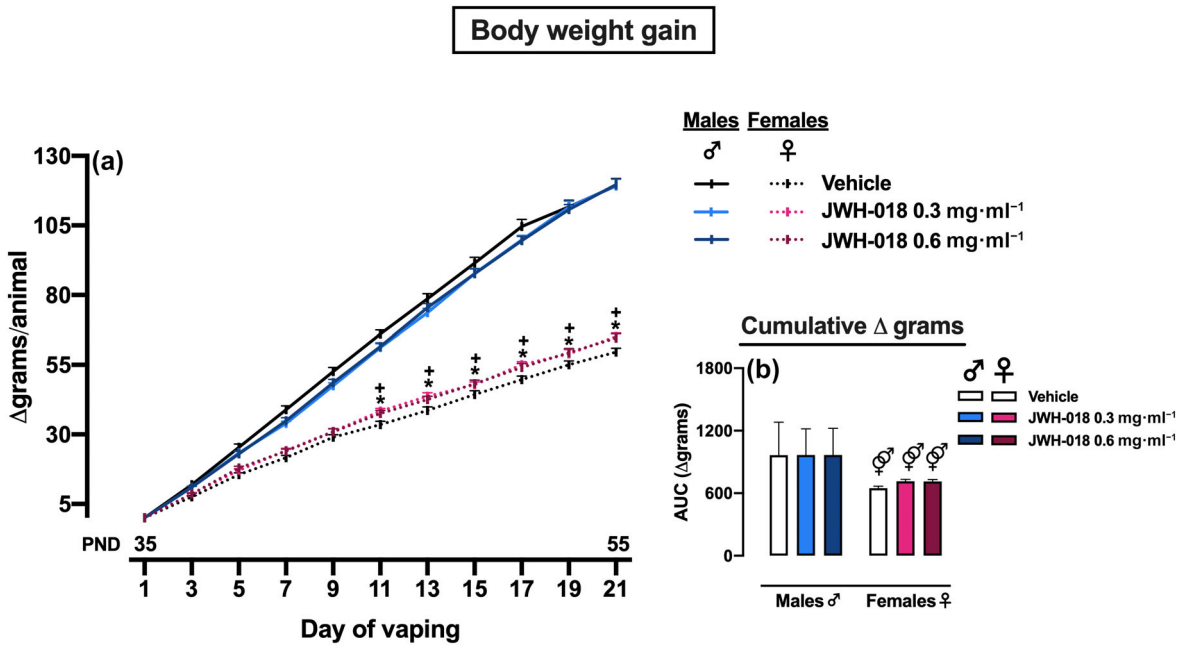

FIGURE 3 Repeated JWH-018 inhalation during adolescence exerted sex-dependent effects on body weight gain. Data are presented as mean ± SEM of increment (Δ grams) of body weight (BW), expressed as daily (a) and cumulative (measured by areas under the curves, AUC) ΔBW (b) showed by male and female rats during the vaping period (postnatal day [PND] 35–55, 21 days). *$P < 0.05$ vehicle (Veh) versus JWH-018 0.3 mg·ml^{-1} within sex; + $P < 0.05$ Veh versus JWH-018 0.6 mg·ml^{-1} within sex (two-way ANOVA, Tukey's post hoc test), ♂ $P < 0.05$ males versus females (two-way ANOVA, Sidak's post hoc test). Male: Veh, JWH-018 0.6 mg·ml^{-1} n = 18 per group; JWH-018 0.3 mg·ml^{-1} n = 20. Female: Veh n = 20; JWH-018 0.3 and 0.6 mg·ml^{-1} n = 22 per group.

3.2.2 | Locomotor activity

As shown in Figure 4, JWH-018 inhalation induced sex-specific locomotor sensitization. In males, repeated but not acute inhalation of JWH-018 affected locomotion in a dose and time-dependent fashion, whereas in females it induced transient hypolocomotion independent of the dose. Noteworthy, sex differences in total ambulation pre- and post-inhalation testing period were observed at each time point, with females exhibiting higher locomotion than males (Figure S2a–f; see statistical details in Table S4), except for the JWH-018 0.3-mg·ml^{-1} group after Days 14 and 21 of vaping (Figure S2g,h). To better appreciate the effect of treatment in each sex, data were analysed separately. In males, two-way ANOVA of total ambulatory counts post-inhalation showed a main effect of treatment [$F_{(2,15)} = 8.99$, $P < 0.01$], session [$F_{(3,45)} = 21.80$, $P < 0.0001$], and a session × treatment interaction [$F_{(6,45)} = 6.56$, $P < 0.0001$]. Tukey's post hoc test revealed increased locomotion of the JWH-018 0.3-mg·ml^{-1} group as compared to the Vehicle (Days 14–21) and JWH-018 0.6 groups (Figure 4a). In contrast, repeated JWH-018 0.6-mg·ml^{-1} inhalation induced hypolocomotion, as compared to Vehicle (Day 7) and JWH-018 0.3-mg·ml^{-1} (Days 7–14) groups, followed by hyperlocomotion on Day 21 as compared to Vehicle group (Figure 4a). Dose-specific differences in locomotion were also observed during the first 15-min post-inhalation testing. Specifically, JWH-018 0.3 mg·ml^{-1} group exhibited higher locomotion as compared to Vehicle (Days 14–21) and JWH-018 0.6 mg·ml^{-1} (Days 7–21) groups, while JWH-018 0.6-mg·ml^{-1} group exhibited higher locomotion on Day 21 as compared to Vehicle group (Two-way ANOVA: treatment × time interaction: 7th day, $F_{(14,105)} = 2.02$, $P < 0.05$; 14th day, $F_{(14,105)} = 2.82$, $P < 0.01$; 21st day, $F_{(14,105)} = 6.37$, $P < 0.0001$; Figure S3a–h). No differences in total ambulatory counts during the pre-inhalation testing period were observed (Figure S2a–d). In females, two-way ANOVA of total ambulatory counts post-inhalation showed a main effect of treatment [$F_{(2,18)} = 5.42$, $P < 0.05$], session [$F_{(3,54)} = 14.07$, $P < 0.0001$] and a session × treatment interaction [$F_{(6,54)} = 2.81$, $P < 0.05$]. Tukey's post hoc test revealed reduced locomotion in both JWH-018 0.3- and 0.6-mg·ml^{-1} groups as compared to the Vehicle group (Days 7–14), and in the JWH-018 0.3 mg·ml^{-1} group on Day 21 as compared to JWH-018 0.6 mg·ml^{-1} group (Figure 4b). No significant differences in locomotor activity during pre-inhalation (total) and post-inhalation (time course of each session) testing sessions were observed (Figures S2a–d and S3i–p).

3.2.3 | Emission of 22- and 50-kHz ultrasonic vocalizations (USVs)

As shown in Figure S4, no significant differences in the emission of 22- and 50-kHz USVs were observed in both sexes during pre- and post-inhalation testing sessions.

3.3 | Behavioural effects of JWH-018 inhalation 24 h after treatment cessation

Repeated JWH-018 inhalation during adolescence induced spontaneous somatic withdrawal signs and negative affect in a sex-dependent manner 24 h post-inhalation period.

As shown in Figure 5a,b, both male and female JWH-018-treated groups exhibited increased spontaneous somatic withdrawal signs 24 h after drug discontinuation as compared to the respective control

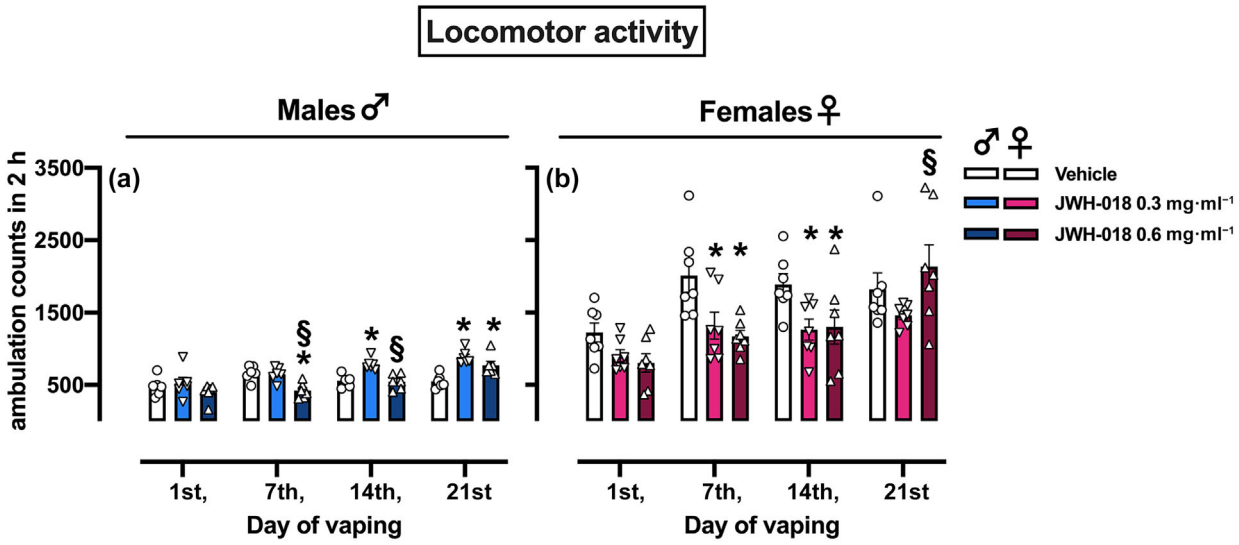

FIGURE 4 Repeated JWH-018 inhalation during adolescence exerted sex-related effects on locomotor activity. Data are presented as individual values with ±SEM of ambulation exhibited by male (a), and female (b) rats, expressed as total ambulatory counts after the 1st, 7th, 14th and 21st vaping session. Repeated measures (RM) two-way ANOVA, Tukey's post hoc test. *$P < 0.05$ versus vehicle (Veh) within sex; § $P < 0.05$ JWH-018 0.3 mg·ml^{-1} versus JWH-018 0.6 mg·ml^{-1} within sex. Males: n = 6 per group; females: n = 7 per group.

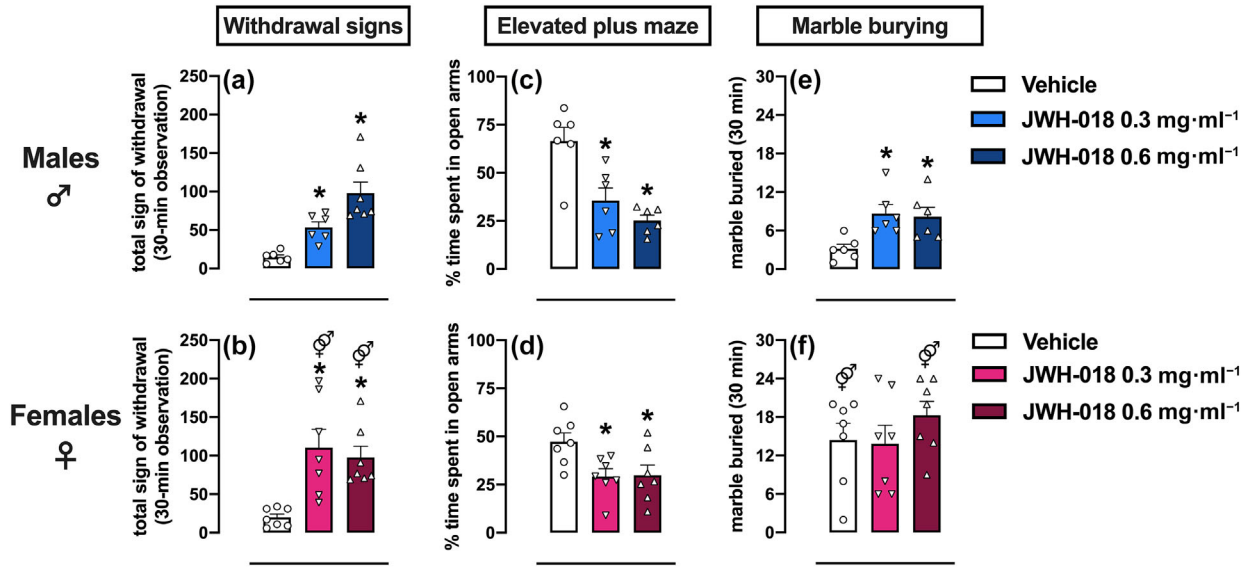

FIGURE 5 Repeated JWH-018 inhalation during adolescence induces spontaneous somatic withdrawal signs and abnormalities of emotional states in a sex-dependent manner 24 h after drug discontinuation. Data are presented as individual values with means ± SEM of total withdrawal scores during a 30-min observation period (a, b), of the percentage of time spent in open arms during the elevated plus maze (EPM) test (c, d) and of the total number of marbles covered with bedding during the marble burying (MB) test (e, f) 24 h after drugs discontinuation exhibited by male and female rats. * $P < 0.05$ versus vehicle (Veh) within sex (one-way ANOVA, Tukey's post hoc test). ♂ $P < 0.05$ males versus females (Two-way ANOVA, Sidak's post hoc test). Males n = 6 per group; females n = 7 per group.

TABLE 2 Spontaneous somatic signs of withdrawal 24 h after JWH-018 discontinuation.

	Males			Females		
Signs of withdrawal 24 h after JWH-018 discontinuation (30-min observation)	**Veh**	**JWH-018 0.3 mg·ml^{-1}**	**JWH-018 0.6 mg·ml^{-1}**	**Veh**	**JWH-018 0.3 mg·ml^{-1}**	**JWH-018 0.6 mg·ml^{-1}**
Facial rubbing	1.5 (0–4)	10.5 (6–17)*	8 (3–19)*	2 (1–6)	11 (6–32)*	11 (1–31)*
Licking	1 (0–2)	13.5 (4–15)*	10.5 (9–35)*	7 (2–20)	22 (8–46)*	20 (7–30)
Ptosis eyelid	0 (0–1)	0.5 (0–15)	0 (0–18)	0 (0–2)	1 (0–25)	1 (0–9)
Wet dog shakes	0	0	0	0 (0–2)	0 (0–2)	0 (0–2)
Arched back	0	0 (0–5)	0.5 (0–3)	0	0 (0–2)	0 (0–2)
Biting	0	0	0	0	2 (0–8)*	3 (0–5)*
Head shakes	0	0 (0–1)	0.5 (0–1)	0	0 (0–6)	0
Chewing	2 (1–5)	5.5 (3–12)	7.5 (2–16)*	3 (0–10)	20 (4–35)*	5 (0–33)
Tongue rolling	6 (1–12)	6.5 (2–17)	10.5 (5–23)	2 (0–5)	12 (1–32)*	9 (1–24)*
Paw treading	0 (0–2)	0	0	0 (0–2)	7 (0–40)*	4 (0–23)
Forepaw fluttering	0 (0–1)	4.5 (1–8)*	0 (0–3)	1 (0–3)	6 (1–32)*	12 (4–22)*
Teeth chattering	0.5 (0–4)	4.5 (0–13)	3 (0–7)	0 (0–1)	5 (0–14)*	4 (0–23)*
Scratching	0 (0–1)	1 (0–1)	0.5 (0–3)	0 (0–1)	7 (0–11)*	5 (1–16)*

Note: Data are expressed as median ± 95% CI of behavioural withdrawal scores exhibited by male and female rats during a 30-min observation 24 h after JWH-018 discontinuation. Males n = 6 per group; females n = 7 per group.
*$P < 0.05$ versus vehicle (Veh) within sex (Kruskal–Wallis test, Dunn's post hoc tests).

groups, with JWH-018 treatment eliciting a greater number of signs in females than males (two-way ANOVA: sex main effect, $F_{[1,33]} = 11.66$, $P < 0.01$). In males, Dunn's post hoc tests revealed an increase in withdrawal signs, such as facial rubbing (K-W test: 11.13; $P < 0.001$), liking (K-W test: 11.47; $P < 0.001$), chewing (K-W test: 7.87; $P < 0.05$) and forepaw fluttering (K-W test: 10.37; $P < 0.01$) in both JWH-018 0.3 and 0.6mg·ml^{-1} groups as compared to the Vehicle group (Table 2). Similarly, in females, Dunn's post hoc tests revealed an increase in facial rubbing (K-W test: 9.11; $P < 0.01$), licking (K-W test: 7.95; $P < 0.05$), chewing (K-W test:8.89; $P < 0.01$), forepaw fluttering (K-W test: 11.92; $P < 0.001$), along with increased biting (K-W test: 8.02; $P < 0.05$), tongue rolling (K-W test: 7.68;

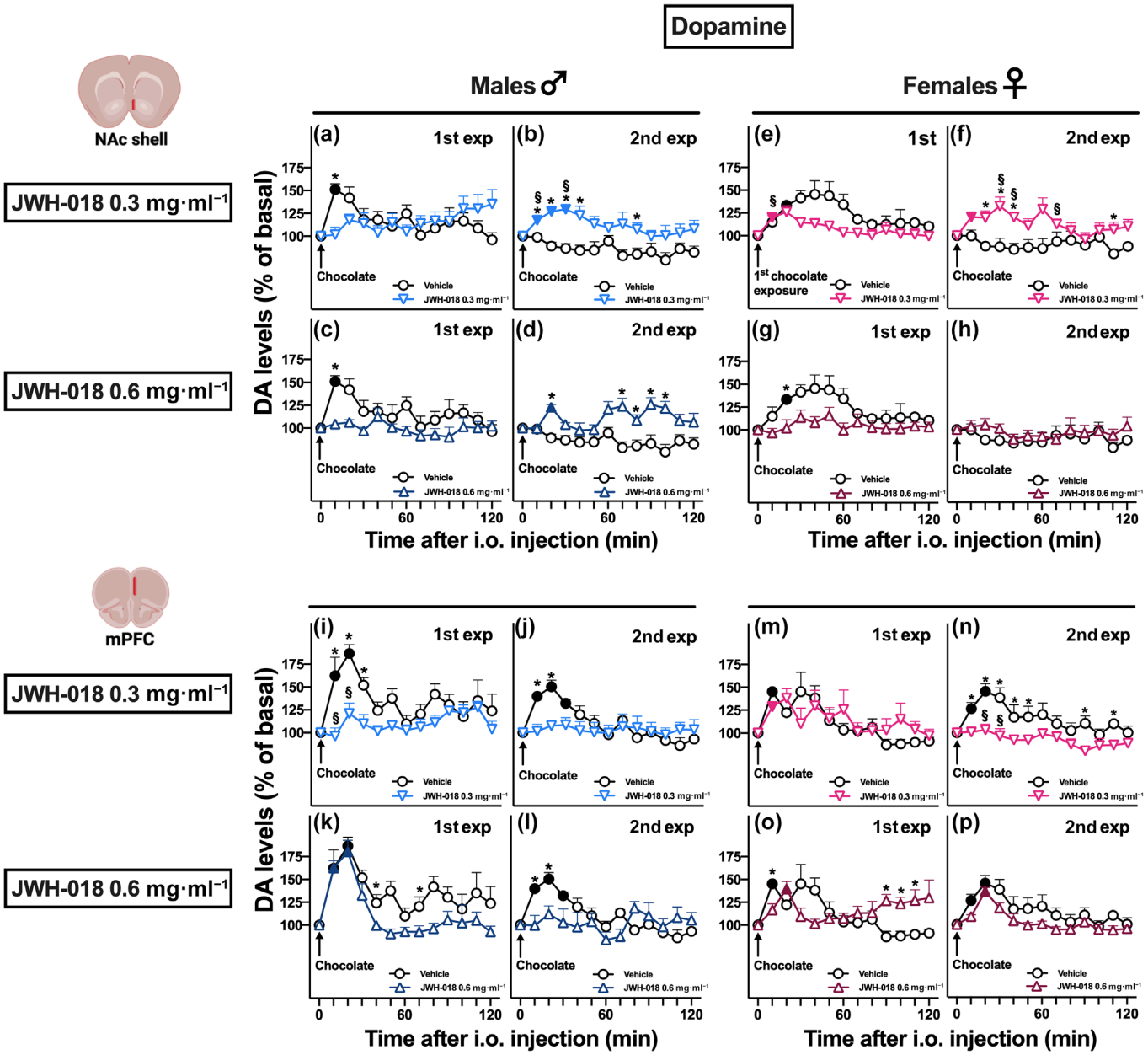

FIGURE 6 Legend on next page.

FIGURE 6 Adolescent JWH-018 inhalation alters NAc shell and mPFC DA responsiveness to chocolate in a dose- and sex-dependent manner in early adulthood. Data are presented as mean ± SEM of changes in extracellular levels of dopamine, expressed as the percentage of basal values. The arrow indicates the start of chocolate intraoral infusion (1 ml/5 min) in vehicle (Veh) (black circle), and JWH-018 0.3- or 0.6-mg·ml^{-1}-treated male (light blue or dark blue triangle, respectively) and female (light pink or dark pink triangle, respectively) rats implanted in the NAc shell (a–h) and in the mPFC (i–p). For clarity, the Veh data are the same in JWH-018 0.3- and 0.6-mg·ml^{-1} graphs, which are separated for clarity of presentation. Repeated measures (RM) two-way ANOVA, Tukey's post hoc test. Solid symbols: $P < 0.05$ versus basal values; * $P < 0.05$ Veh versus JWH-018 within brain area and chocolate exposure; § $P < 0.05$ JWH-018 0.3 mg·ml^{-1} versus JWH-018 0.6 mg·ml^{-1} within brain area and chocolate exposure. Males: first exposure NAc shell and mPFC, Veh, JWH-018 0.6 mg·ml^{-1} n = 6 per group, JWH-018 0.3 mg·ml^{-1} n = 8; second exposure NAc shell, Veh, JWH-018 0.6 mg·ml^{-1} n = 6 per group, JWH-018 0.3 mg·ml^{-1} n = 7; second exposure mPFC: Veh n = 6, JWH-018 0.3 mg·ml^{-1} n = 8, JWH-018 0.6 mg·ml^{-1} n = 5. Females: first exposure NAc shell, Veh n = 6, JWH-018 0.3 and 0.6 mg·ml^{-1} n = 8 per group; first exposure mPFC, Veh n = 6, JWH-018 0.3 mg·ml^{-1} n = 7, JWH-018 0.6 mg·ml^{-1} n = 8; second exposure NAc shell, Veh n = 6, JWH-018 0.3 and 0.6 mg·ml^{-1} n = 8 per group; second exposure mPFC, Veh, JWH-018 0.3 mg·ml^{-1} n = 6 per group, JWH-018 0.6 mg·ml^{-1} n = 8.

$P < 0.05$), paw treading (K-W test: 7.72; $P < 0.05$), teeth chattering (K-W test: 9.70; $P < 0.01$) and scratching (K-W test: 11.33; $P < 0.01$) in both JWH-018 0.3- and 0.6-mg·ml^{-1} groups as compared to the Vehicle group (Table 2).

Concurrently, repeated JWH-018 inhalation induced sex-dependent affective disturbances. Both sexes exposed to each JWH-018 dose spent less time in the open EPM arms (One-way ANOVA: male, $F_{(2,15)} = 13.26$; $P < 0.001$; female, $F_{(2,18)} = 4.87$; $P < 0.05$, Figure 5c,d). However, only male JWH-018-treated groups buried a higher number of marbles in the MB test as compared to the Vehicle group (one way-ANOVA: $F_{(2,15)} = 6.08$; $P < 0.05$, Figure 5e,f). Notably, female rats exposed to Vehicle and JWH-018 0.6 mg·ml^{-1} buried a higher absolute number of marbles than males (two-way ANOVA: sex main effect, $F_{[1,33]} = 23.25$, $P < 0.0001$; Figure 5e,f).

3.4 | Effects of adolescent JWH-018 inhalation on dopamine (DA) responsiveness and taste reactions to repeated chocolate exposure in early adulthood

As shown in Figure 6, JWH-018 inhalation during adolescence altered NAc shell and mPFC DA responsiveness and taste reactions to chocolate exposure in early adulthood, in a sex- and dose-dependent manner. Specifically, in male rats, inhalation of each dose of JWH-018 abolished DA responsiveness in the NAc shell to the first chocolate exposure while increasing DA release on the second exposure (Figure 6a–d). In contrast, in female rats, the lower dose of JWH-018 increased DA responsiveness in the NAc shell to each chocolate exposure, while the higher dose blunted NAc shell DA responsiveness to chocolate (Figure 6e–h).

On the other hand, in male rats, inhalation of the lower JWH-018 dose led to a loss of DA responsiveness to chocolate in the mPFC, while in females, the same dose induced a decrease of mPFC DA responsiveness (habituation) to the second chocolate exposure (Figure 6i,j,m,n). Habituation of mPFC DA responsiveness was also observed in male rats exposed to the higher JWH-018 dose, while in females, this dose had no significant effect on DA responsiveness (Figure 6k,l,o,p). Besides these changes in DA responsiveness, JWH-018 inhalation during adolescent induced sex-specific changes in taste reactions to chocolate exposure (Figure 7).

3.4.1 | NAc shell and mPFC DA responsiveness

No differences in basal DA levels in the NAc shell and in the mPFC between sexes and among groups were observed before each chocolate exposure (Figure S5).

In the NAc shell of males, two-way ANOVA of the first chocolate exposure showed a significant time × treatment interaction [$F_{(36,264)} = 2.82$, $P < 0.0001$]. Tukey's post hoc test revealed an increase of dialysate DA in the NAc shell of the Vehicle group by the first chocolate exposure as compared to basal values and to both JWH-018 0.3 and 0.6-mg·ml^{-1} groups (10′) (Figure 6a,c). Two-way

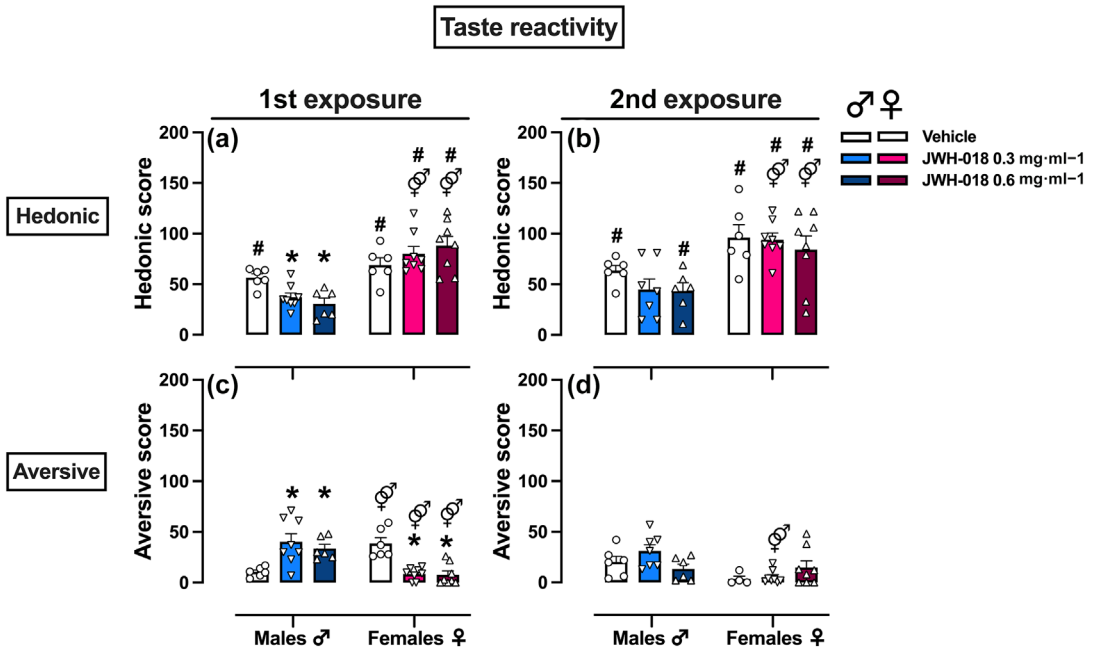

FIGURE 7 Adolescent JWH-018 inhalation alters taste reactions to chocolate in a sex-dependent manner in early adulthood. Data are presented as individual values with ±SEM of behavioural hedonic (a, b) and aversive (c, d) scores to the first and the second chocolate exposure. *$P < 0.05$ versus vehicle (Veh) within sex and chocolate exposure (one-way ANOVA, Tukey's post hoc test); ♂ $P < 0.05$ males versus females within chocolate exposure; # $P < 0.05$ hedonic versus aversive within sex and treatment (two-way ANOVA, Sidak's post hoc test). Males: Veh, JWH-018 0.6 mg·ml^{-1} n = 6 per group, JWH-018 0.3 mg·ml^{-1} n = 8; females: Veh n = 6, JWH-018 0.3 and 0.6 mg·ml^{-1} n = 8 per group.

ANOVA of the second chocolate exposure showed a main effect of treatment [$F_{(2,16)} = 10.85$, $P < 0.01$], a time × treatment interaction [$F_{(24,192)} = 3.08$, $P < 0.0001$]. Tukey's post hoc test revealed an increase of dialysate DA in the NAc shell of both JWH-018 0.3- and 0.6-mg·ml^{-1} groups by the second chocolate exposure as compared to basal values (0.3 mg·ml^{-1}:10′–30′; 0.6 mg·ml^{-1}: 20′), and to Veh group (0.3 mg·ml^{-1}: 10′–40′, 80′; 0.6 mg·ml^{-1}: 20′–30′, 70′–100′), and differences in dialysate DA between JWH-018-treated groups (0.3 mg·ml^{-1} > 0.6 mg·ml^{-1}: 10′, 30′, Figure 6b,d).

In the NAc shell of females, two-way ANOVA of the first chocolate exposure showed a main effect of treatment [$F_{(2,19)} = 4.24$, $P < 0.05$], time [$F_{(5.92,112.5)} = 5.80$, $P < 0.0001$] and a time × treatment interaction [$F_{(24,228)} = 1.91$, $P < 0.01$]. Tukey's post hoc test revealed an increase of dialysate DA in the NAc shell of Veh and JWH-018 0.3-mg·ml^{-1} groups by the first chocolate exposure as compared to basal values and to the JWH-018 0.6-mg·ml^{-1} group (Veh: 20′; 0.3 mg·ml^{-1}: 10′) (Figure 6e,g). Two-way ANOVA of the second chocolate exposure showed a main effect of treatment [$F_{(2,19)} = 6.78$, $P < 0.01$], a time × treatment interaction [$F_{(24,228)} = 1.57$, $P < 0.05$]. Tukey's post hoc test revealed an increase of dialysate DA in the NAc shell of JWH-018 0.3-mg·ml^{-1} group by the second chocolate exposure as compared to basal values (10′), to Veh (20′- 40′, 110′) and to JWH-018 0.6-mg·ml^{-1} (30′–40′, 70′) groups (Figure 6f,h).

In the mPFC of males, two-way ANOVA of the first chocolate exposure showed a main effect of time [$F_{(5.21,88.58)} = 12.83$, $P < 0.0001$] and treatment [$F_{(2,17)} = 4.87$, $P < 0.05$] and a time × treatment interaction [$F_{(24,204)} = 4.02$, $P < 0.0001$]. Tukey's post hoc test revealed an increase of dialysate DA in the mPFC of Veh and JWH-018-0.6-mg·ml^{-1} groups by the first chocolate exposure as compared to basal values (Veh: 20′–30′; 0.6 mg·ml^{-1}: 10′–20′) and to the JWH-018 0.3-mg·ml^{-1} group (Veh: 10′–30′; JWH-018 0.6 mg·ml^{-1}: 10′–20′), and differences in dialysate DA between Vehicle and JWH-018 0.6-mg·ml^{-1} groups (Veh > JWH-018: 50′–80′, Figure 6i,k). Two-way ANOVA of the second chocolate exposure showed a main effect of time [$F_{(5.73,91.63)} = 3.27$, $P < 0.01$], and a time × treatment interaction [$F_{(24,192)} = 2.47$, $P < 0.001$]. Tukey's post hoc test revealed an increase of dialysate DA in the mPFC of Veh group by the second chocolate exposure as compared to basal values (10′–30′) and to both JWH-018 0.3- and 0.6-mg·ml^{-1} groups (10′–20′, Figure 6j,l).

In the mPFC of females, two-way ANOVA of the first chocolate exposure showed a main effect of time [$F_{(5.52,99.42)} = 3.27$, $P < 0.01$] and a time × treatment interaction [$F_{(24,216)} = 2.30$, $P < 0.001$], but not of treatment. Tukey's post hoc test revealed an increase of dialysate DA in the mPFC of Vehicle and both JWH-018-treated groups by the first chocolate exposure as compared to basal values (Vehicle and JWH-018 0.3 mg·ml^{-1}: 10′; JWH-018 0.6 mg·ml^{-1}: 20′), and differences in dialysate DA between Vehicle and JWH-018 0.6-mg·ml^{-1} groups (10′, 90′–110′) (Figure 6m,o). Two-way ANOVA of the second chocolate exposure showed a main effect of time [$F_{(5.78,98.23)} = 11.03$, $P < 0.0001$], treatment [$F_{(2,17)} = 9.12$, $P < 0.01$] and a time × treatment interaction [$F_{(24,204)} = 1.58$, $P < 0.05$]. Tukey's post hoc test revealed an increase of dialysate DA in the mPFC of Veh and

JWH-018 0.6-mg·ml^{-1} groups by the second chocolate exposure as compared to basal values (20′) and to the JWH-018 0.3-mg·ml^{-1} group (Veh: 10′–30′,90′,110′; JWH-018 0.6-mg·ml^{-1}:10′–20′, 90′) (Figure 6n,p).

Summing up, control male as well as female rats, given two sequential intraoral sweet chocolate infusions 4 h apart, showed a stimulatory DA response in the NAc shell to the first but not to the second chocolate infusion (habituation). No such habituation was observed in the mPFC. Rats withdrawn by 1 week from daily JWH-018 vapour showed sex-, dose- and time-dependent changes in the responsiveness of NAc shell and mPFC DA to intraoral chocolate infusion. Thus, the NAc shell DA response to the first chocolate infusion was abolished in males withdrawn from both doses of JWH-018 and in females exposed to the higher dose of JWH-018, while it was increased in females exposed to the lower dose of JWH-018. As to the second chocolate infusion, a stimulatory DA response, not seen in controls, emerged in males withdrawn from both JWH-018 doses and in females exposed to the lower dose of JWH-018. In females withdrawn from higher JWH-018, no response was observed, as in control rats. As to the mPFC DA response to intraoral chocolate, no increase was the prevalent feature of males, except for a stimulatory response to the first chocolate infusion in the higher JWH-018 dose group. In females exposed to JWH-018, mPFC DA increased in response to chocolate, except for no response to the second chocolate infusion in the lower JWH-018 dose group.

3.4.2 | Taste reactions

Female rats exposed to each JWH-018 dose exhibited a greater number of hedonic reactions during both chocolate exposures than males (two-way ANOVA sex main effect: first chocolate exposure, $F_{(1,36)} = 46.70$, $P < 0.0001$; second chocolate exposure, $F_{(1,35)} = 23.62$, $P < 0.0001$; Figure 7a,b). Moreover, female rats exposed to each JWH-018 dose exhibited lower aversive reactions during the first chocolate exposure as compared to males, this effect was also observed during the second chocolate exposure only in the JWH-018 0.3-mg·ml^{-1} group (Two-way ANOVA sex main effect: first chocolate exposure, $F_{(1,36)} = 5.74$, $P < 0.05$; second chocolate exposure, $F_{(1,35)} = 10.53$, $P < 0.01$; Figure 7c,d). Notably, differences in aversive reactions during the first chocolate exposure between male and female Vehicle rats were observed (female > male; Figure 7c).

To better understand the effect of treatment on taste reactions in each sex, the data were analysed separately. In males, one-way ANOVA revealed that both JWH-018 0.3- and 0.6-mg·ml^{-1} groups showed a decrease in hedonic and an increase in aversive score during the first chocolate exposure as compared to the Vehicle group (hedonic: $F_{(2,17)} = 7.63$, $P < 0.001$; aversive: $F_{(2,17)} = 6.50$, $P < 0.01$; Figure 7a,c). No differences in hedonic and aversive scores among groups were observed during the second chocolate exposure (Figure 7b,d). Differently, in females, both JWH-018 0.3- and 0.6-mg·ml^{-1} groups showed a decrease in the aversive score during the first chocolate exposure as compared to the Vehicle group (K-W test: 12.59, $P < 0.001$; Figure 7c). No differences in hedonic (first and second chocolate exposure) and aversive (second chocolate exposure) scores among female groups were observed (Figure 7a,b,d). Noteworthy, all female groups exhibited higher hedonic reactions during both chocolate exposure as compared to aversive score (two-way ANOVA reactions main effect: first chocolate exposure, $F_{(1,38)} = 139.4$, $P < 0.0001$; second chocolate exposure, $F_{(1,36)} = 121.4$, $P < 0.0001$). A similar effect was observed in male Vehicle (first and second chocolate exposure) and JWH-018 0.6-mg·ml^{-1} (second chocolate exposure) groups (two-way ANOVA reactions main effect: first chocolate exposure, $F_{(1,34)} = 8.80$, $P < 0.01$; second chocolate exposure, $F_{(1,32)} = 23.74$, $P < 0.0001$).

4 | DISCUSSION

The main finding of this study is that repeated inhalation of the full CB receptor agonist JWH-018 during adolescence induced sex-specific behavioural effects and physical dependence and, following abstinence, sex-dependent adaptive changes in the responsiveness of NAc shell and mPFC DA transmission and behavioural reactivity to a taste reward (intraoral chocolate solution) in early adulthood in rats. We also observed sex differences in the JWH-018 plasma pharmacokinetic profile after single and repeated inhalation. Taken together, these results demonstrate that JWH-018 inhalation during adolescence in rats induces a broad spectrum of sex-specific effects across adolescence and adulthood that recapitulate signs of cannabinoid dependence in rodents, highlighting the role of sex in the vulnerability of the adolescent brain to SCRA.

Regarding the impact of JWH-018 vapour exposure during adolescence in rats on the effect of two sequential intraoral chocolate infusions on the NAc shell and mPFC DA transmission, sex- and dose-dependent changes were observed. Specifically, in male rats, each JWH-018 dose tested altered the pattern of NAc shell DA responsiveness observed in control, abolishing the DA increase to the first chocolate exposure and increasing DA response to the second one, that is not present in controls as a result of 'habituation' (Bassareo et al., 2002; De Luca, 2014; Pintori et al., 2021), but is consistent with previous findings observed in adult rats repeatedly exposed to JWH-018 (Pintori et al., 2021). Unlike the female control rats that, likes males, exhibited a stimulatory DA response to the first chocolate infusion that 'habituates' on the second one, JWH-018-treated groups showed different adaptation of the NAc shell DA response to chocolate. In particular, the lower JWH-018 dose increased the NAc shell DA response to both chocolate exposure, indicative of a loss of habituation, whereas the higher JWH-018 dose abolished it. These sex-dependent differences in NAc shell DA responsiveness may confirm a higher sensitivity of female rats to cannabinoid effects. For example, female rats acquire self-administration of the synthetic cannabinoid WIN 55,212-2 more readily than males (L. Fattore et al., 2007), and oestradiol enhances the reinforcing effects of cannabinoids in females (Craft et al., 2013). Sex- and dose-specific changes in DA responsiveness to intraoral chocolate were also observed in the mPFC. In male

rats, the lower JWH-018 dose abolished DA responsiveness, whereas in females, the same dose induced habituation. Habituation of mPFC DA response was also observed in males exposed to the higher JWH-018 dose, while in females, this dose had no significant effect. Consistent with our previous study (Pintori et al., 2021), lower concentrations of JWH-018 vapour induced adaptive changes of DA responsiveness to chocolate, with a decrease of DA responsiveness in the mPFC and an increase in the NAc shell. These opposite outcomes are consistent with the differential responsiveness of NAc shell and mPFC DA to rewarding stimuli (Bassareo et al., 2002; De Luca, 2014). It is well known that the endocannabinoid (eCB) system can influence VTA DA neurons, modulating excitatory and inhibitory inputs. For instance, adolescent THC exposure impaired GABA and glutamate balance in the mPFC and induced a hyper-dopaminergic state of VTA DA neurons, along with cognitive and emotional alterations (Renard, Rosen, et al., 2017; Renard, Szkudlarek, et al., 2017). Moreover, a persistent decrease of VTA DA neuron activity and CB_1 receptor down-regulation in the mPFC and NAc were observed after repeated JWH-018 exposure in adulthood (Pintori et al., 2021). Therefore, influencing eCB system function and neurodevelopment processes, adolescent JWH-018 inhalation may alter the activity of VTA DA neurons involved in rewarding, emotional and cognitive processes. Taken together, these findings demonstrated that repeated JWH-018 inhalation during adolescence induced stronger alterations of DA responsiveness to motivational taste stimuli than previously observed in adults (Pintori et al., 2021), confirming the higher sensitivity of adolescent mesocorticolimbic DA system to SCRAs.

We also observed sex-specific changes in taste reactions to intraoral chocolate, especially to the first infusion, with males exhibiting increased aversive and decreased hedonic reactions, suggesting an aversive state, while females showed decreased aversive reactions. These differential—even opposite—behavioural changes may be correlated to sexual dimorphism and sex-dependent JWH-018 pharmacokinetic profiles, and in turn, to the sex-specific impact of JWH-018 on emotional and cognitive processes. Although aversive taste reactions, as that observed in males, has long been associated with dopaminergic dysfunctions, reactions to taste stimuli does not directly depend on dopamine (Bassareo et al., 2002; Bassareo & Di Chiara, 1999; Berridge, 1991, 2007; De Luca, 2014). Therefore, the contemporary increase of aversive and decrease of hedonic reactions to novel taste stimulus observed in male rats might represent an aversive and neophopic state, may be due to persistent abnormalities of emotional state induced by JWH-018 inhalation, consistently with acute withdrawal findings and those observed in adults rats repeatedly exposed to JWH-018 (Pintori et al., 2021). Consistent with the literature and the influence of oestrous cycle in the taste reactivity response (Contini et al., 2018), females showed greater hedonic taste reactions to chocolate than aversive. For instance, female rats exhibited more hedonic reactions to sucrose than male rats, particularly during the dioestrus or pro-oestrus phases (Clarke & Ossenkopp, 1998). In addition, since the eCB system has a prominent influence on the hedonic effects of natural rewards, such as food (Parsons & Hurd, 2015; Silvestri & Di Marzo, 2013), dysregulation of eCB signalling, as those potentially induced by JWH-018 inhalation, might be involved in the sex-specific alterations of taste reactivity observed.

We observed sex-dependent plasma pharmacokinetic profiles of JWH-018 and its first-pass metabolite (JWH-018 5-hydroxyindole) after JWH-018 vapour inhalation. After a single inhalation of each dose, males exhibited higher plasma concentrations of JWH-018 than females. Similarly, after the repeated inhalation (21 sessions) of JWH-018, males exhibited higher plasma JWH-018 concentrations compared to females and to the concentrations observed after a single inhalation. Notably, in both sexes, there was no linearity in peak plasma levels following the double JWH-018 concentration—1.68 and 2.83 $ng{\cdot}ml^{-1}$ in males, and 1.20 and 1.69 $ng{\cdot}ml^{-1}$ in females after JWH-018 0.3- and 0.6-$mg{\cdot}ml^{-1}$ inhalation, respectively. This unexpected lack of linearity may be related to technical and methodological limits; indeed, since the plasma collection started only after 35 min from the beginning of vaping session (5 min after the end of inhalation), it is possible that we missed important steps of JWH-018 pharmacokinetics, such as absorption, distribution and metabolism, that could be influenced by JWH-018 concentration.

Consistently, repeated JWH-018 inhalation induced sex-dependent differences in plasma accumulation of JWH-018, with male rats exhibiting higher levels than females before the final inhalation session, that is, 48 h after the previous session. This sex-specific accumulation phenomena could be in part due to sex differences in body weight observed during the vaping period (males > females), and given the lipophilicity of these compounds, in the amount of adipose tissue which may passively and slowly diffuse back JWH-018 into blood. Sex-specific differences were also observed for the JWH-018 5-hydroxyindole metabolite, a full CB_1 receptor agonist (Brents et al., 2011). At the lower dose (0.3 $mg{\cdot}ml^{-1}$), accumulation of the 5-hydroxyindole metabolite was observed in males but not in females. Moreover, JWH-018 5-hydroxyindole levels trended to higher in males than females after a single inhalation of each dose, with males also reaching the peak concentration later than females. Marked and opposite changes in Tmax of 5-hydroxyindole metabolite—reduction in males and increase in females—were observed after repeated JWH-018 inhalation, suggesting sex-specific changes in JWH-018 metabolism. In both sexes, independently of the dose and number of inhalations, the 5-hydroxyindole metabolite levels were two to three times lower than the parent compound. Consistent with human and animal evidence (Kevin et al., 2017; Toennes et al., 2017), our findings suggest a slow metabolism and clearance of JWH-018, likely due to sequestration into adipose tissue, as confirmed by the accumulation observed after repeated inhalation.

Few studies investigated SCRAs pharmacokinetics in male rats (Kevin et al., 2017; Uttl et al., 2018), and only one in mice of both sexes (Corli et al., 2024). In contrast to our results, Corli and colleagues (2023) reported higher—approximately double—plasma AKB48 levels in adult female mice than males following single (6 $mg{\cdot}kg^{-1}$, i.p.) and repeated (6 $mg{\cdot}kg^{-1}$, i.p., weekly, 3 weeks) exposure, in line with the literature on THC (Narimatsu et al., 1991; Tseng et al., 2004). These discrepancies may stem from differences in species (rats vs. mice), development stage (adolescent vs. adults), dosing

regimens (0.3–0.6 mg·ml^{-1} vs. 6 mg·kg^{-1}) and administration routes (inhalation vs. i.p.). Nevertheless, in line with our findings and in vitro SCRA inhibitory activity on cytochrome P-450 enzymes (Ashino et al., 2014; Kim et al., 2020), a rise of plasma AKB48 levels at each drug administration was observed in both sexes, correlating with sex-specific behavioural (i.e., sensorimotor responses and nociception) outcomes (Corli et al., 2024).

We found sex-specific alterations in body weight gain and locomotion throughout the JWH-018 inhalation period. Females, but not males, exposed to each dose exhibited an increased body weight gain from Day 11 of inhalation up to the treatment cessation. These data align with the role of the eCB system in modulating feeding, metabolism and energy balance (Matias et al., 2008; Matias & Di Marzo, 2007), as well as sex differences in cannabinoid-regulated processes (Fattore & Fratta, 2010; Wagner, 2016). In contrast with our results, several studies reported either no differences (Bruijnzeel et al., 2019) or a reduction (Lin et al., 2023; Scherma et al., 2016) in body weight gain with adolescent THC exposure. Considering SCRAs, only two studies reported weight loss in adult male rats during repeated HU-210 exposure that correlated with brain CB_1 receptor down-regulation (Dalton et al., 2009; Giuliani et al., 2000). Besides pharmacokinetics, sex dimorphism in the pharmacodynamics of eCB system (e.g., differences in receptor number and activity) and its interaction with other mediators involved in food intake and energy homeostasis, such as leptin and orexin, might explain our findings.

Concurrently with body weight gain, repeated JWH-018 inhalation affected locomotion in a sex-dependent manner. In males, the lower JWH-018 dose induced a behavioural sensitization to JWH-018, since rats showed a progressive hyperlocomotion after 14 and 21 days of inhalation, whereas the higher dose tested produced a biphasic effect: an initial hypolocomotion (Day 7) followed by hyperlocomotion at the later session. In contrast, females exposed to JWH-018 exhibited transient hypolocomotion after 7 and 14 days of inhalation that disappeared after 21 days, possibly as a result of initial behavioural sensitization. These biphasic effects are consistent with the ability of natural (THC) and synthetic cannabinoids (WIN 55,212.2) to induce behavioural sensitization in rodents (Cadoni et al., 2001; Cadoni et al., 2008; Enayatfard et al., 2013; Rubino et al., 2001), an effect associated with adaptive changes of DA transmission in NAc shell versus core. The higher sensitivity to JWH-018-induced hypolocomotion and its subsequent tolerance observed in females, a classical effect observed after chronic CB_1 agonists (Maldonado & de Rodríguez Fonseca, 2002), is consistent with the literature reporting that female rodents are more sensitive to cannabinoid-induced locomotor effects (Craft et al., 2013) and shows higher CB_1 receptor density, with earlier peak expression (Rodríguez de Fonseca et al., 1993) and quicker desensitization (Burston et al., 2010) than males.

Conversely, JWH-018 inhalation did not stimulate the emission of either 22- or 50-kHz USVs in both sexes during the inhalation period. Crucially, as 22-kHz USVs are a reliable ethological indicator of negative affective states (e.g., stress, aversive state and discomfort) (Brudzynski, 2013) that may also index the presence of affective discomfort following drug withdrawal (Covington & Miczek, 2003), the absence of a pre- and post-inhalation increase in 22-kHz USVs in all experimental groups may eliminate potential confounding factors, such as withdrawal effects between sessions or stressful procedural stimuli (i.e., vapour exposure), in the observed effects.

Consistent with sexual dimorphism, females exposed to JWH-018 exhibited a greater number and variety of somatic withdrawal signs 24 h after JWH-018 cessation, potentially linked to their distinct plasma accumulation profile. Additionally, both sexes exhibited increased anxiety-like behaviours in the EPM, an effect that was associated only in male rats with higher repetitive-like behaviours in the MB test. Consistent with our previous findings on adult rats (Pintori et al., 2021), adolescent JWH-018 inhalation altered emotional state during the acute withdrawal phase, which may reflect dysregulations of the eCB system, a key modulator of anxiety-related responses (Parolaro et al., 2010).

Taken together, the present findings support the hypothesis that cannabinoid inhalation during adolescence disrupts the neurodevelopment trajectories of mesocorticolimbic eCB and DA circuitry in a sex-dependent manner, increasing the risk of developing dependence and neuropsychiatric disorders in adulthood.

5 | LIMITATIONS

This study was limited to evaluating profiles of two selected first-pass JWH-018 metabolites (see also SI). Additionally, pharmacokinetic assessments were only performed in the plasma. Future studies should extend these analyses to brain tissue and include additional metabolites to provide a more comprehensive pharmacokinetic profile. Moreover, the interpretation body weight changes is constrained by the absence of food intake and growth data. Another limitation derives from the lack of oestrous cycle phase assessments. Finally, ad hoc molecular studies are needed to understand the underlying mechanisms of behavioural and neurochemical effects, as well as explain the sex differences observed in this study.

6 | CONCLUSIONS

Our findings demonstrate that repeated JWH-018 inhalation during adolescence induces sex-specific behavioural and neurochemical alterations across adolescence and adulthood. Importantly, this study highlights the key role of sex in modulating JWH-018 effects in adolescent rats. Along with sexually dimorphic pharmacokinetic properties, JWH-018 altered body weight gain only in females, induced stronger withdrawal signs in females than males, and induced sex- and dose-dependent dysregulation of locomotion and DA responsiveness to motivational stimuli. Using a model of administration with high translational value, our findings confirmed the role of sex in the vulnerability of the adolescent brain to SCRAs and the importance of further research into gender-specific pharmacology and toxicology.

AUTHOR CONTRIBUTIONS

N. Pintori: Conceptualization; data curation; formal analysis; investigation; writing—original draft; writing—review and editing. **C. Manis:** Investigation. **E. Spano:** Investigation. **N. Simola:** Investigation. **A. Ieraci:** Investigation. **P. Caboni:** Investigation. **G. Di Chiara:** Writing—review and editing. **M. A. De Luca:** Conceptualization; funding acquisition; supervision; writing—original draft; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis and Animal Experimentation and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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