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I. TITLE

REPEATED EXPOSURE TO JWH-018 INDUCES ADAPTIVE CHANGES IN THE MESOLIMBIC AND MESOCORTICAL DOPAMINE PATHWAYS, GLIAL CELLS ALTERATION AND BEHAVIOURAL CORRELATES

II. RUNNING TITLE

Running title: Protracted administration of synthetic cannabinoids receptor agonist

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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.

V. WORD COUNT

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TOSSICODIPENDENTI NEGLI ISTITUTI PENITENZIARI DELLA SARDEGNA” (Resolution of the Special Commissioner ATS n. 121 of 21-02-2020).

VII. CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

VIII. ABSTRACT

BACKGROUND AND PURPOSE

Spice/K2 herbal mixtures, containing synthetic cannabinoids such as JWH-018, have been marketed as marijuana surrogates since 2004. We demonstrated that JWH-018 has cannabinoid CB1 receptor-dependent reinforcing properties and acutely increases dopamine transmission selectively in the NAc shell. Here we tested the hypothesis that repeated administration of JWH-018 (i) modulates behaviour, (ii) affects dopamine transmission and its responsiveness to motivational stimuli, and (iii) is associated with a neuroinflammatory phenotype.

EXPERIMENTAL APPROACH

Rats were administered with JWH-018 once a day for 14 consecutive days, then we performed behavioural, electrophysiological, and neurochemical evaluation at multiple time points after drug discontinuation.

KEY RESULTS

Our data demonstrated that repeated JWH-018 exposure (i) induces anxious and aversive behaviours, transitory attentional deficits and withdrawal signs, (ii) decreases spontaneous activity and number of dopamine neurons in the VTA and (iii) reduces the stimulation of dopamine transmission in the NAc shell while potentiating that in the NAc core in response

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to acute JWH-018 challenge. Moreover, (iv) we observed a decreased dopamine sensitivity in the NAc shell and core, but not in the mPFC, to the first chocolate exposure; conversely, after the second exposure, dialysate dopamine fully increased in the NAc shell and core but not in the mPFC. Finally, selected dopamine brain areas showed (v) astrogliosis (mPFC, NAc shell and core, VTA), microgliosis (NAc shell and core), and downregulation of CB1Rs (mPFC, NAc shell and core).

CONCLUSION AND IMPLICATIONS

These results suggest that repeated JWH-018 exposure may reflect a useful model to clarify the detrimental effects of recurring use of Spice/K2 drugs.

ABBREVIATIONS

Δ9-THC, Δ9-Tetrahydrocannabinol; CBRs, Cannabinoid receptors; CPu, Caudate putamen; DA, Dopamine; eCB, Endocannabinoid; EPM, Elevated plus maze; GFAP, Glial fibrillary acidic protein; GLT-1, Glutamate transporter-1; Glu, Glutamate; HPLC, High performance liquid chromatography; IBA-1, Ionized calcium-binding adapter molecule-1; IR, Immunoreactivity; JWH-018, 1-pentyl-3-(1-naphthoyl) indole; NAc, Nucleus accumbens; NPS, New psychoactive substances; MB, Marble burying; mPFC, medial prefrontal cortex; PBS, Phosphate buffer solution; PPI, Prepulse inhibition; PSB, Pontamine sky blue; SC, Synthetic cannabinoid; SCRA, Synthetic cannabinoid receptor agonist; SD, Sprague-Dawley; TH, Tyrosine hydroxylase; VTA, Ventral tegmental area.

IX. KEYWORDS

Addiction, dopamine, glial cells, habituation, novel psychoactive substances, synthetic cannabinoids, taste

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X. MAIN TEXT

INTRODUCTION

Novel psychoactive substances (NPS) are a broad variety of drugs not controlled by the United Nations drug conventions. The advent of NPS has contributed to the appearance and growth of a new 'drug scenario' characterised by an increased number of drug users among youth and consumption of drugs with unknown effects and safety profiles. The first wave of NPS came to Europe in the early 2000s when herbal mixtures containing synthetic cannabinoids (SC), broadly known as *Spice/K2 drugs*, have been marketed as legal marijuana surrogates. JWH-018 (1-pentyl-3-(1-naphthoyl) indole) is a SC receptor agonist (SCRA), highly potent and efficacious at CB1 and CB2 receptors (CB1Rs and CB2Rs), that has been detected in several *Spice/K2 drugs* (Wiley, Marusich, Martin, & Huffman, 2012). Initially developed for therapeutic purposes, JWH-018 is considered the prototypical compound of the so-called 'first-generation' class of SC. Thereafter, newer generations of SC arose from slight modifications to JWH-018 chemical structure, generating legal drugs with higher potency and efficacy at CB1Rs and CB2Rs and increased abuse liability (De Luca & Fattore, 2018). Compared to Δ-9-tetrahydrocannabinol (THC), SCRAs induce more severe adverse reactions and psychiatric consequences (Papanti, Orsolini, Francesconi, & Schifano, 2014; Schifano, Orsolini, Duccio Papanti, & Corkery, 2015), likely due to their action as full CB1Rs agonist (De Luca et al., 2016; Pintori, Loi, & Mereu, 2017). Previously, we demonstrated that JWH-018 elicits CB1R-dependent reinforcing properties and dopamine (DA) stimulant actions preferentially on the nucleus accumbens (NAc) shell at doses 4-fold lower (0.25mg/kg ip) than THC (De Luca et al., 2015).

The role of mesolimbic and mesocortical DA transmission in salient information and reward processing is well known (Di Chiara et al., 2004). In particular, drugs of abuse and

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natural rewarding stimuli, as highly appetitive taste stimuli, increase DA transmission in the NAc shell (Bassareo, De Luca, & Di Chiara, 2002; Di Chiara, 1990; Volkow, Fowler, & Wang, 2003). However, the neurochemical effect of taste stimuli undergoes habituation, i.e. adaptive regulation, following repeated exposure (Bassareo et al., 2002; Di Chiara, 1990). Importantly, we previously showed that this phenomenon requires an intact DA function in the medial prefrontal cortex (mPFC) (i.e. infralimbic and prelimbic cortices), since bilateral lesions of these regions abolished *habituation* of NAc shell DA to repeated chocolate exposure (Bimpisidis, De Luca, Pisanu, & Di Chiara, 2013). These and other data (De Luca, Bimpisidis, Bassareo, & Di Chiara, 2011) support the notion of top-down control of mPFC on NAc DA transmission and its putative role in the loss of control of the motivational value of stimuli (De Luca, 2014; Goldstein & Volkow, 2011). The endocannabinoid (eCB) system and DA transmission strictly interact in the evaluation of salience information and reward (Manzanares et al., 2018; Sagheddu, Muntoni, Pistis, & Melis, 2015; Tan, Ahmad, Loureiro, Zunder, & Laviolette, 2014; Volkow, Hampson, & Baler, 2017). Indeed, dysfunctions of eCB signalling may lead to the dysregulation of mesolimbic and mesocortical DA transmission, which is associated with several neuropsychiatric disorders, including addiction (Martin-Santos et al., 2010; Tan et al., 2014; Volkow et al., 2003; Volkow et al., 2017; Volkow & Morales, 2015). Besides neurons, accumulating evidence strongly supports an important contribution of glial cells in brain plasticity and in affective, motivational and cognitive processes (Marin & Kipnis, 2013; Yirmiya & Goshen, 2011). Important glial alterations produced by different addictive drugs, including THC, have been observed (Lacagnina, Rivera, & Bilbo, 2017; Melis et al., 2017; Scofield & Kalivas, 2014; Secci et al., 2019). Despite the widespread and growing use of *Spice/K2 drugs*, limited information is available on the effects induced by repeated exposure to SCRAs on mesolimbic and mesocortical DA transmission and glial cells. To investigate these aspects in more depth, we evaluated the

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neurochemical and behavioural modifications occurring in adult male Sprague-Dawley rats repeatedly administered with JWH-018. The dose able to selectively increase DA transmission in the NAc shell (0.25mg/kg ip) was selected on the basis of our previous studies, lower and higher doses being ineffective on DA release (De Luca et al., 2015). At multiple time-points after JWH-018 discontinuation (1h, 24h or 7 days), we performed a battery of behavioural tests to evaluate the occurrence of anxiety-, aversive-, compulsive-like behaviours, attentional dysfunction, and spontaneous somatic withdrawal signs. In addition, changes in ventral tegmental area (VTA) DA neurons activity during JWH-018 withdrawal were recorded and compared to the tyrosine hydroxylase (TH)-immunoreactivity (IR) in the same area. Furthermore, in order to explore whether or not a loss of control of the motivational value of stimuli can be induced by repeated exposure to JWH-018, the pattern of DA responses to a natural rewarding stimulus (i.e. intraoral chocolate) was estimated by *in vivo* microdialysis in the NAc shell and core and in the mPFC. Moreover, the levels of markers of glia activation, such as glial fibrillary acidic protein (GFAP) and ionised calcium-binding adapter molecule-1 (IBA-1), were measured in the mPFC, NAc, and VTA. Finally, changes in CB1Rs expression were evaluated in selected DA terminal (i.e. mPFC and NAc). Taken together, these preclinical studies are useful to elucidate the severe consequences of the recurring use of Spice/K2 drugs with the ultimate goal to help clinicians to manage synthetic cannabinoid intoxications and related use disorders.

MATERIALS AND METHODS

Animals

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 Ethical Committee for Animal Experiments (CESA,

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University of Cagliari). We made all efforts to minimise pain and suffering, and to reduce the number of animals used. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010) and with the recommendations made by the British Journal of Pharmacology (BJP). The total number of animals as well as their suffering was minimised, according to the 3R principle. Adult male Sprague-Dawley (SD, 275-300g) rats (Envigo, Italy) were housed in groups of six at temperature of 22±2 °C and 60% humidity, under a 12 h light/dark cycle (lights on at 7.00 a.m.) and with *ad libitum* access to water and food (Mucedola, Italy).

Drugs and solutions

JWH-018 was purchased from Tocris (Bristol, UK) and solubilised in 0.5% EtOH, 0.5% Tween 80 and 99% saline. A solution containing chocolate syrup (Nesquik Squeeze©, Nestle, Switzerland) and tap water (1:1) was used as a gustatory taste stimulus. A chocolate solution was administered twice in order to evaluate rats' taste responses to either a single or repeated exposure to a salient stimulus.

Experimental design timeline

Rats were injected once a day with either JWH-018 (0.25mg/kg) or vehicle intraperitoneally (ip) for 14 consecutive days. Animals were assigned randomly to different experimental groups for behavioural, electrophysiological, neurochemical and molecular evaluation at different time points (1h, 24h or 7 days) after drug discontinuation (Figure 1). The experimental group sizes (n≥5) were chosen based on our previous experimental protocols (Castelli et al., 2014; Sagheddu et al., 2020; Zanda et al., 2017) and are reported in the figure legends. Due to technical issues (e.g., catheter or dialysis probes obstruction) some animals were excluded from statistical analysis thus reducing the group size in few cases. Data evaluation and analysis were performed by blinded experimenters.

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Behavioural tests

Separate groups of animals were used at different time-points for each of the following behavioural tests:

Elevated Plus Maze (EPM). The EPM is a simple method for assessing spatial anxiety in rodents (Morales et al., 2010). In the present study, the EPM paradigm was used to evaluate possible anxiogenic effects in rats induced by repeated JWH-018 administration and JWH-018 withdrawal syndrome (Morales et al., 2010). On each evaluation day, rats were placed for a 2h period of acclimation into the experimental room. The EPM was made of white PVC and consisted of two opposite open arms (length 50 cm, width 10 cm) and two opposite closed arms (length 50 cm, width 10 cm), the latter enclosed by 40 cm high walls along their length. The four arms converged to a central square (10×10 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 illumination of 40 lux, which was uniform in both the open and closed arms of the apparatus. Rats' performance was videotaped and later evaluated to calculate the percentages of arm entries and of time spent in open and closed with respect to the total number of entries and to the total amount of time spent in the arms. A rat was considered inside a specific arm when it had all four paws inside that arm.

Marble Burying test (MB). The MB is used to evaluate compulsive activity/repetitive like-behaviours in rodents (Zanda et al., 2017). Before starting the evaluation, rats were placed for a 2h period of acclimation into the experimental room. The MB was conducted into an open transparent plastic cage (54×34.5×20cm) with 5 cm of fresh hardwood chip bedding as previously described (Satta et al., 2016). Twenty-four standard glass marbles (1.5 cm in diameter, arranged in six rows of four marbles each) were placed uniformly over the bedding

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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 was counted as previously described (Zanda et al., 2017). New bedding was used for each animal, and marbles were cleaned with soap and tap water in between each evaluation.

Prepulse Inhibition (PPI). The PPI was performed to evaluate attentional deficits induced by repeated JWH-018 exposure or drug withdrawal. On the day of the experiment, rats were placed for a 2h period of acclimation into the experimental room. The startle reflex system consisted of 4 standard cages each placed inside a sound-attenuated and ventilated chamber (Med Associated, USA). Startle cages were non-restrictive Plexiglas cylinders (diameter 9 cm) mounted on a piezoelectric accelerometer platform connected to an analogue-digital converter. Background noise and acoustic bursts were conveyed through two speakers placed in proximity to the startle cage, to produce a variation in sound intensity within 1 dB. On test day, each rat was placed in the experimental cage for a 5-min acclimation period with a 70 dB white noise background; this was continued for the remainder of the session. Animals were then tested on 3 consecutive trial blocks. The first and the third blocks consisted of 5 pulse-alone trials of 40 ms at 115 dB, while the second block (test block) was a pseudorandom sequence of 50 trials including 12 pulse-alone trials, 30 pulse trials preceded by 73, 76 or 82 dB prepulses (10 for each level of prepulse loudness), and 8 no-stimulus trials (where the only background noise was delivered). The percentage of PPI was calculated based only on the values relative to the second block, and using the following formula: 100-[(mean startle amplitude for prepulse + pulse trials/mean startle amplitude for pulse-alone trials) × 100] (Spano, Fadda, Frau, Fattore, & Fratta, 2010).

Spontaneous somatic signs of withdrawal. Rats were individually placed in plastic cages (30×25×45 cm) with standard rat bedding. Cages were located in a sound-proof room for

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behavioural observation. Point-scoring was performed by an observer (placed behind a one-way window) blind to the treatment. The spontaneous cannabinoid withdrawal signs 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, Melis, Muntoni, & Gessa, 1998).

Taste reactivity test. During the 5-min intraoral chocolate infusion, animals were monitored and two classes of taste reactivity patterns were scored, i.e. 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 (Bassareo et al., 2002; De Luca, Solinas, Bimpisidis, Goldberg, & Di Chiara, 2012). Each reaction was scored and assigned one point if it lasted 1-5s and two points if it lasted more than 5s.

***In vivo* electrophysiology: VTA DA neuron single unit recordings**

Twenty-four hours or seven days after JWH-018 discontinuation, rats were anaesthetised with urethane (1.3 g/kg, ip) and placed in a stereotaxic apparatus (Kopf, Tujunga, USA) with their body temperature maintained at 37±1°C by a heating pad. The scalp was retracted, and one small hole was drilled above the parabrachial pigmented nucleus (PBP) of the posterior VTA according to the Rat Brain Atlas (Paxinos & Watson, 2007) coordinates (A: -5.8 to -6.2 from bregma, L: +0.4 to +0.6 from midline, V: -7.0 to - 8.0 from the cortical surface). Extracellular single-unit activity was recorded with glass micropipettes filled with 2% pontamine sky blue (PSB) dissolved in 0.5 M sodium acetate (impedance 2.5–5 MΩ). The spontaneous population activity was determined by descending the electrode within the area in 6-9 predetermined tracks separated by 200μm each other. Putative VTA DA neurons were selected when all criteria for identification were fulfilled: firing rate <10 Hz and duration of action potential >2.5 ms as measured from start to end (Grace & Bunney, 1984). Bursts were defined as the occurrence of two spikes at interspike interval <80 ms, and terminated when

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the interspike interval exceeded 160ms (Grace & Bunney, 1984). The electrical activity for each neuron was recorded for 2-3 min and filtered (bandpass 0.1–10,000Hz). Individual action potentials were isolated and amplified (Neurolog System, Digitimer, UK) and displayed on a digital storage oscilloscope (TDS 3012, Tektronics, UK). Experiments were sampled on line with Spike2 software (Cambridge Electronic Design, UK) by a computer connected to CED 1401 interface (Cambridge Electronic Design, UK). At the end of recording sessions, DC current (15mA for 1min) was passed through the recording micropipette in order to eject PSB for marking the recording site. Brains were then rapidly removed and frozen in isopentane cooled to -40°C. The position of the electrodes was microscopically identified on serial 60µm sections stained with Neutral Red (Sagheddu et al., 2019).

Preparation of microdialysis probe and oral catheters

Vertical microdialysis probes, with an active dialysing portion of 1.5 mm for NAc and 3 mm for mPFC, were prepared with AN69 fibers (Hospal Dasco, Italy) as previously described (De Luca et al., 2015). The oral catheters were made of a 22-G stainless steel needle and polyethylene (PE) tubing (Portex Ltd, Hythe, England) (ID 0.58mm, OD 0.96mm) as previously described (Bimpisidis et al., 2013). The needle was cut at one side (total length of 2cm from the tip), the cut part was blunted and inserted in the PE tubing, which ended with a perforated circular disc.

Chocolate exposure

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 22G needle, and fixed on the top of the head of the rat with a small plastic tip filled with cyanoacrylate glue.

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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, to a total of 1 mL/5min (Bimpisidis et al., 2013; De Luca et al., 2011).

***In vivo* microdialysis**

Surgery. Rats were anaesthetised 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 core (A:+1.4; L:+1.6 from bregma; V:-7.6 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).

Dopamine assessment. On the day after surgery, probes were perfused with Ringer's solution (in mM: 147 NaCl, 4 KCl, 2.2 $CaCl_2$) at a constant rate of 1µl/min. Dialysate samples (10µl) were injected into an HPLC equipped with a reverse phase column (C8 3.5 um, Waters, USA) and a coulometric detector (ESA, Coulochem II) to quantify DA as described previously (De Luca et al., 2015). The first electrode of the detector was set at +130 mV (oxidation) and the second at -175 mV (reduction). The composition of the mobile phase was (in mM): 50 NaH_2PO_4, 0.1 Na_2-EDTA, 0.5 n-octyl sodium sulphate and 15% (v/v) methanol; pH 5.5. The sensitivity of the assay for DA was 5fmoles/sample. After 2h washing, DA basal levels were evaluated, estimated as the mean of three consecutive samples whose values did not differ more than 10%.

Glutamate and GABA assessment. Two days after surgery, probes were perfused with Ringer's solution at a constant rate of 2.2µl/min. Glutamate (Glu) and GABA were analysed by HPLC coupled with laser-induced fluorescence detection. Dialysate samples were stored at -80°C until processed when each sample (20µl) was added to 500mM borate buffer (pH 8.7, 20µl), 10mM KCN in borate buffer (120µl), and 5mM naphtalene dicarboxaldehyde (NDA) (Fluka, Germany) in CH_3OH (20µl), the solution was vortex-mixed and left 4 min at

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room temperature for derivatisation. Then, 15µl were injected into the HPLC that consisted in a Waters 515 pump (Waters, Italy), an Agilent 1200 series autosampler (Agilent Technologies, Germany), and a Zetalif detector (Picometrics S.A. France) coupled with a He-Cd laser (Melles-Griot, USA) working at 442nm. Mobile phase A was 50mM sodium acetate (pH 5.5 with glacial acetic acid) and CH_3CN (J.T. Baker, The Netherlands), 77% and 23% respectively; mobile phase B was 50mM sodium acetate/CH_3CN (40:60). Separation was performed with a 3.0×150mm, C18 (3.5µm) Symmetry column (Waters, Italy) at 100% mobile phase A for 25 min, 100% B for 8 min and 100% A for the last 10 min. Before use, mobile phase was filtered through 0.45µm MF Millipore filter (Millipore, USA) and degassed under vacuum in an ultrasonic bath. Flow rate was set at 1ml/min and column temperature was maintained at 28°C by a Series 1100 thermostat (Agilent Technologies, Germany). Fluorescence was measured setting detector range at 20 Relative Fluorescence Units (RFU). After standard curve calibration, the area of the peaks was quantified by means of a Waters Millennium computer program. After 2h washing, Glu and GABA basal levels were evaluated as the mean of three consecutive samples whose values did not differ more than 10%.

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

Immunohistochemical assay

Twenty-four hours or 7 days after the last JWH-018 injection, rats were deeply anaesthetised with Equithesin and then transcardially perfused with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M phosphate-buffered solution, pH 7.4. Brains were rapidly removed and post-fixed in the same fixative overnight. After repeated washing in 0.1M PBS, brains were cryoprotected in 30% sucrose in PBS for 48h. Immunostaining was performed on free-

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floating coronal sections (40μm) which were obtained using a cryostat at levels comprising the brain areas selected for this study according to the Rat Brain Atlas coordinates (Paxinos & Watson, 2007). In particular, sections containing mPFC (PrL and IL cortex; AP: +3.70 to +2.70), NAc (NAc shell and core; AP: +1.20 to +0.70), and VTA (AP: -4.80 to -6.30) were processed for GFAP and IBA-1 immunoreactivity (IR). CB1R-IR analysis was performed in the sections containing the mPFC and NAc, while TH-IR analysis only in the sections containing the VTA. All immunohistochemical procedures and analyses comply with the recommendations detailed in the BJP editorial and adhere to the BJP checklist for immunohistochemistry (S. P. H. Alexander et al., 2018).

GFAP immunofluorescence. Tissue sections were incubated at 4C° for 24h with a mouse monoclonal IgG anti-GFAP antibody (1:5000; Millipore Cat# MAB360, RRID:AB_11212597) in PBS containing 0.2% Triton X-100, 0.1% BSA and 1% NGS. Then, sections were washed in PBS containing 0.2% Triton X-100 and incubated with Alexa Fluor 594-labeled goat anti-mouse IgG (1:500; cat n° A-11005, Molecular Probes, USA) for 1h in the dark at room temperature (Castelli et al., 2014).

IBA-1 immunofluorescence. Tissue sections were incubated at 4°C for 24h with a rabbit polyclonal IgG anti-IBA-1 antibody directed against synthetic peptide corresponding to C-terminus of Iba1 (1:2000; FUJIFILM Wako Shibayagi Cat# 016-20001, RRID:AB_839506) in PBS containing 0.2% Triton X-100, 0.1% BSA, and 1% NGS. Then, sections were washed in PBS containing 0.2% Triton X-100 and incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG (1:500; cat n° A-11012 Molecular Probes, USA) for 1h in the dark at room temperature.

TH immunofluorescence. Tissue sections were incubated at 4°C for 24h with a mouse monoclonal IgG anti-TH antibody directed against an epitope on the outside of the regulatory N-terminus (1:1000; Millipore Cat# MAB318, RRID:AB_2201528) in PBS containing 0.2%

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Triton X-100, 0.1% BSA, and 1% NGS. Then, sections were washed in PBS containing 0.2% Triton X-100 and incubated with Alexa Fluor 488-labeled goat anti-mouse IgG (1:500; cat n° A-28175 Molecular Probes, USA) for 1h in the dark at room temperature. Finally, all sections were rinsed and mounted on slides using VectaShield anti-fade mounting media (Vector Inc.).

CB1R immunofluorescence. Tissue sections were incubated at 4°C for 48h with rabbit anti-CB1 receptor polyclonal antibody directed against the last 15 amino acids of rat CB1R (1:2000) (Bodor et al., 2005) in PBS containing 0.2% Triton X-100, 2,5% BSA, and 10% NGS, kindly supplied by Dr K. Mackie (Indiana State University, USA). Then, sections were washed in PBS containing 0.2% Triton X-100 and incubated with biotinylated goat anti-rabbit IgG (1:200, cat n° BA-1000, Vector Laboratories, Burlingame, CA, USA) for 1h in the dark at room temperature. Subsequently, sections were incubated with Avidin Alexa Fluor 488 (1:1000, cat n° A-21370, Molecular Probes, USA) for 1h in the dark at room temperature, then rinsed and mounted on slides using VectaShield anti-fade mounting media (Vector Inc.).

Standard control experiments were performed by omitting either the primary or secondary antibody and yielded no cellular labelling (data not shown). All the diluting buffers and the final antibody dilutions were used only once.

Imaging and quantitative analysis of IBA-1, TH, GFAP and CB1R immunofluorescent staining

An Olympus IX 61 microscope and an Olympus 12-bit cooled F View II camera (Hamburg, Germany) were used for observations and for capturing the images, respectively. For each animal, analysis of IBA-1- IR and GFAP-IR were performed on one tissue section out of every 3 successive sections, for a total of 8, 6, and 12 sections containing the mPFC, the NAc

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shell and core, and the VTA respectively. CB1R-IR analysis was performed in the sections containing the mPFC and the NAc shell and core, while TH-IR analysis only in sections containing the VTA.

IBA-1 and TH analysis. The total size of the examined area in which IBA-1 and TH neurons were counted was chosen according to the extension of the region under analysis, in order to include almost the whole area (either mPFC, NAc, VTA). The number of both IBA-1 and TH positive cells was counted bilaterally in the different sections per animal. In these sections, non-overlapping randomly selected region of interest (ROIs) of 0.15mm^2, 6 and 4 respectively for IBA-1 and TH, were examined with a 20 X objective by two trained observers blind to drug treatment. Limits of the ROI were defined based on structural details within the tissue sections to ensure the ROIs did not overlap. The distance among the 6/4 ROIs was superior to 40μm to avoid overlapping. IBA-1 and TH positive cells touching the inferior or the right sides of the ROI were excluded from counting. The number of IBA-1 and TH cells was expressed as mean/mm^2±SEM.

GFAP and CB1R analysis. The semi-quantitative analysis of GFAP-IR and CB1R -IR was carried out on three non-overlapping areas (ROIs, roughly 140,000 and 16,000 μm^2 for GFAP and CB1R, respectively) from tissue slices of each brain region using the 20X and 60X objective for GFAP- and CB1R-IR respectively. The focus depth was extended by summing the maximum intensity of several images taken at focus steps of 0.25 μm-depth intervals to a total of 2 μm thicknesses using the Z-stack module (Olympus soft Imaging solution, GNHB, Germany). Images were analysed using the Cell P AnalySIS software module. Positively stained fibers were detected by means of density thresholding applied to the single-channel greyscale images. Average values of GFAP and CB1R-IR were calculated from images per brain region of each animal and were expressed as average values of percentage of area occupied by fibers.

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Statistical analysis

Data and statistical analysis complies with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). Normality tests for data were carried out using Shapiro-Wilk's test. If data were found to be normally distributed, the effect of treatment was analysed using Student's t-test (EPM, basal DA extracellular levels, cells/track index, firing rate, TH-IR experiments). If data were found to not be normally distributed and/or there was significant variance inhomogeneity, the effect of treatment was analysed using Mann-Whitney test (MB, spontaneous somatic withdrawal signs, PPI, basal Glu and GABA extracellular levels, % of spikes/bursts, aversive taste reactions).

The effect of treatment on DA responses to JWH-018 challenge and chocolate exposures was analysed by repeated measures (RM) two-way ANOVA (treatment x time) followed by Bonferroni's multiple comparisons. 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). The time course of DA response in each group (data not normally distributed) was analysed by nonparametric RM one-way ANOVA (Friedman's test) followed by Dunn's multiple comparisons. The effect of JWH-018 on GFAP, IBA-1, and CB1R-IR was analysed by two-way ANOVA (treatment × brain area) followed by Bonferroni's multiple comparisons. Then, the effect of treatment on GFAP, IBA-1, and CB1R-IR within each brain areas was analysed by Student's t-test. *Post-hoc* tests were conducted only if F in ANOVA achieved $P<0.05$ and there was no significant variance inhomogeneity (Brown-Forsythe's test). In this study, P values <0.05 were considered as statistically significant. Statistical analysis was performed with Statistica (StatSoft, Tulsa, OK, USA) or GraphPad Prism 8 (GraphPad Prism, RRID:SCR_002798) software. Numerical data are given as mean±SEM for parametric analysis or as median with 95% Cl for non-parametric analysis.

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Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2017), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Stephen P H Alexander et al., 2019).

RESULTS

Effect of JWH-018 repeated exposure in behavioural tests at multiple time-points after drug discontinuation

In order to disclose whether JWH-018 repeated exposure induced behavioural abnormalities, such as anxiety-like state, compulsive-like behavioural activity, attentional deficits, and spontaneous signs of withdrawal, we performed a battery of behavioural tests (EPM, MB, PPI) at multiple time-points after JWH-018 discontinuation (1h, 24h or 7 days). Behavioural observation of spontaneous somatic withdrawal signs has been performed at 24h or 7 days after JWH-018 discontinuation.

Elevated Plus Maze

One hour and 24h after drug discontinuation, rats treated with JWH-018 spent less time in the open arms of the EPM with respect to Veh-treated group (1h: $t_{(14)}=2.73$, $P<0.05$; 24h: $t_{(14)}=2.15$, $P<0.05$; Figure 2a,e); no significant differences between groups were observed 7 days after drug discontinuation (Figure 2i) as well as in the total number of arm entries (data not shown).

Marble Burying test

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One hour, 24h and 7 days after drug discontinuation, rats treated with JWH-018 buried a higher number of marbles with respect to Veh-treated group (1h, U:6.0, P<0.05; 24h, U:7.5, P<0.05; 7 days, U:6.5, P<0.05; Figure 2b,f,j).

Prepulse Inhibition

Rats treated with JWH-018 did not display significant differences in startle amplitude with respect to Veh-treated group at any time point. However, rats treated with JWH-018 displayed a significant reduction of average % PPI levels 1h after drug discontinuation (U:12 P<0.05; Figure 2d). No significant differences were observed at both 24hs and 7 days after the last JWH-018 administration (Figure 2h,l).

Spontaneous somatic signs of withdrawal

Twenty-four hours after drug discontinuation, JWH-018-treated rats exhibited a significant increase in signs of withdrawal such as licking (U:44, P<0.05), headshakes (U:42, P<0.05), teeth chattering (U:45.5, P<0.05), biting (U:33.5, P<0.05), chewing (U:27.5, P<0.05), and tongue rolling (U:29.5, P<0.01) with respect to Veh-treated group (Table 1). At 7 days after drug discontinuation, JWH-018-treated rats exhibited a significant increase only in biting (U:18, P<0.05) with respect to Veh-treated group (Table 1).

Effect of JWH-018 repeated exposure on DA neuronal activity and TH-IR in the VTA at 24h and 7 days after drug discontinuation

In order to disclose whether JWH-018 repeated exposure induced alteration of basal DA neurons function, we evaluated 24h and 7 days after JWH-018 discontinuation the spontaneous DA neuronal activity as well TH-IR in the VTA.

As showed in Figure 3, repeated JWH-018 exposure induces adaptive changes of VTA DA neuronal activity and of TH-IR in the same area. Twenty-four hours after JWH-018 discontinuation, Student's t-test showed a significant decrease of the number of

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spontaneously active DA cells in JWH-018-treated as compared to Veh-treated group ($t_{(19)}$=2.21, $P<0.05$; Figure 3a). No significant difference in the average firing rate or percentage of spikes/bursts was observed between groups (Figure 3b). Seven days after JWH-018 discontinuation, Student's t-test showed a significant decrease in the number of spontaneously active DA cells in JWH-018-treated as compared to Veh-treated group ($t_{(14)}$=1.85, $P<0.05$; Figure 3e). The average firing rate was significantly lower in JWH-018-treated than in Veh-treated group ($t_{(222)}$=1.94, $P<0.05$; Figure 3f), whereas no significant differences in the percentage of spikes in bursts were observed between groups (Figure 3g). Moreover, the number of TH-positive cells in the VTA both at 24h ($t_{(8)}$=2.82, $P<0.05$; Figure 3d) and at 7 days ($t_{(8)}$=2.82, $P<0.05$; Figure 3h) was significantly reduced after JWH-018 discontinuation.

Effect of JWH-018 repeated exposure on extracellular basal DA, GABA and Glu levels in the NAc and mPFC at 24h and 7 days after drug discontinuation

In order to disclose whether JWH-018 repeated exposure induced neurochemical alteration *in vivo*, we evaluated 24h and 7 days after JWH-018 discontinuation the basal DA extracellular levels (mean of three consecutive samples whose values did not differ more than 10%) in the NAc and in the mPFC. Meantime, we evaluated also extracellular basal Glu and GABA levels in the mPFC, since cortical Glu and GABAergic transmission, both directly and indirectly, regulate mesolimbic and mesocortical DA pathways (Murase, Grenhoff, Chouvet, Gonon, & Svensson, 1993; Renard, Szkudlarek, et al., 2017).

Dopamine

Twenty-four hours after JWH-018 discontinuation, basal extracellular levels of DA, expressed as fmoles/10µl sample (mean±SEM), were: JWH-018 treated group, NAc shell 33±6, NAc core 53±6, mPFC 12±1; Veh-treated group NAc shell 31±7, NAc core 57±13,

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mPFC 18±2. Student's t-test showed reduced basal DA levels in the mPFC of JWH-018-treated compared to Veh-treated group ($t_{(11)}=3.07$, $P<0.05$), while no significant differences were observed in the NAc (Figure 4a). Seven days after JWH-018 discontinuation, basal extracellular levels of DA were: JWH-018 treated group, NAc shell 34±6, NAc core 40±8, mPFC 11±1; Veh-treated group NAc shell 38±5, NAc core 34±6, mPFC 1±1. No significant differences in basal DA levels between groups were observed (Figure 4b).

Glutamate

Twenty-four hours after JWH-018 discontinuation, basal extracellular levels of Glu in the mPFC, expressed as µmoles/10µl sample (mean±SEM) were: JWH-018 treated group 0.6±0.1; Veh-treated group 2.1±0.5. Mann-Whitney test showed a reduced basal Glu extracellular levels in the mPFC of JWH-018-treated compared to Veh-treated group (U:2, $P<0.05$; Figure 4c). Seven days after JWH-018 discontinuation, basal extracellular levels of Glu in the mPFC were: JWH-018 treated group 1.2±0.3; Veh-treated group 1.6±0.4. No significant differences in basal Glu levels between groups were observed (Figure 4d).

GABA

Twenty-four hours after JWH-018 discontinuation, basal extracellular levels of GABA in the mPFC, expressed as nmoles/10µl sample (mean±SEM), were: JWH-018 treated group 64±6; Veh-treated group 106±10. Mann-Whitney test showed reduced basal GABA extracellular levels in the mPFC of JWH-018-treated compared to Veh-treated group (U:1, $P<0.05$; Figure 4e). Seven days after JWH-018 discontinuation, basal extracellular levels of GABA in the mPFC were: JWH-018 treated group 49±19; Veh-treated group 62±22. No significant differences in basal GABA levels between groups were observed (Figure 4f).

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Effect of JWH-018 repeated exposure on DA in the NAc shell, NAc core and mPFC during the last day of JWH-018 treatment

Afterwards, in order to disclose whether JWH-018 repeated exposure induced further neurochemical alteration, we evaluated the responsiveness of mesolimbic and mesocortical DA transmission to an acute JWH-018 challenge. As showed in Figure 5a the last administration of JWH-018 (0.25mg/kg ip) elicits an increase of extracellular DA in the NAc shell of rats pre-treated with vehicle. On the contrary, only rats pre-treated with JWH-018 showed a long-lasting increase of DA release in the NAc core after the last JWH-018 administration (Figure 5b). No significant effects were observed on DA response in the mPFC (Figure 5c). Two-way ANOVA analysis of NAc shell group showed a main effect of treatment ($F_{(1,11)}$=8.37, $P<0.05$), of time ($F_{(12,132)}$=5.04, $P<0.05$) and a significant treatment × time interaction ($F_{(12,132)}$=1.90, $P<0.05$). Bonferroni's *post hoc* test revealed an increase of dialysate DA in Veh-treated group after JWH-018 administration with respect to basal values (30', Figure 5a). To better evaluate the effect of each treatment, data were analysed separately by Friedman's test that revealed differences in the NAc shell of Veh-treated group (F_R=29.27, df 13,7, $P<0.05$). Dunn's test revealed an increase of dialysate DA after JWH-018 administration with respect to basal values (20'-40', Figure 5a). For the NAc core group, two-way ANOVA showed a main effect of treatment ($F_{(1,10)}$=618.90, $P<0.05$), of time ($F_{(12,120)}$=22.65, $P<0.05$) and a significant treatment × time interaction ($F_{(12,120)}$=9.56, $P<0.05$). Bonferroni's *post hoc* tests revealed a long-lasting increase of dialysate DA in the NAc core of JWH-018 treated group after JWH-018 administration as compared to basal values and to Veh-treated group (20'-120', Figure 5b). On the contrary, no significant differences of dialysate DA in the mPFC between groups were observed (Figure 5c).

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Effect of JWH-018 repeated exposure on the responsiveness of DA transmission to chocolate in the NAc shell, NAc core and mPFC at 7 days after drug discontinuation

Furthermore, in order to disclose whether JWH-018 repeated exposure was able or not to induce a loss of control of the motivational value of stimuli, we evaluated 7 days after JWH-018 discontinuation the pattern of DA responses to repeated exposure to a natural rewarding stimulus (i.e. chocolate solution) in the NAc shell and core and in the mPFC. As showed in Figure 6, withdrawal from JWH-018 repeated exposure increases dialysate DA in the mPFC but not in the NAc shell and core after the 1st exposure to chocolate. Conversely, dialysate DA increased in the NAc shell and core but not in the mPFC after the 2nd exposure to chocolate.

In the NAc shell, two-way ANOVA analysis of 1st chocolate exposure showed a main effect of time ($F_{(12,120)}$=13.55, $P<0.05$), treatment ($F_{(1,10)}$=21.98, $P<0.05$) and a significant time x treatment interaction ($F_{(12,120)}$=3.59, $P<0.05$). Bonferroni's *post hoc* tests revealed an increase of dialysate DA in the NAc shell of Veh- treated group after the 1st chocolate exposure as compared to basal values (20'-40') and to JWH-018 treated group (30') (Figure 6a). Considering the 2nd chocolate exposure, two-way ANOVA analysis showed a main effect of time ($F_{(12,120)}$=3.68, $P<0.05$), treatment ($F_{(1,10)}$= 21.88, $P<0.05$) and a significant time x treatment interaction ($F_{(12,120)}$=2.82, $P<0.05$). Bonferroni's *post hoc* test revealed an increase of dialysate DA in the NAc shell of JWH-018 treated group as compared to Veh- treated group (30'). To better evaluate the time course of DA response in each group, data were analysed separately by Friedman's test that revealed significant differences in Veh-treated group after the 1st chocolate exposure (F_R=48.18, df 13,6, $P<0.05$) and in the JWH-018-treated group after the 2nd chocolate exposure (F_R=31.50, df 13,6, $P<0.05$). Dunn's test revealed an increase of dialysate DA with respect to basal values in the NAc shell of both

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Veh-treated (20’, 1st chocolate exposure) and JWH-018 treated groups (20’-40’, 2nd chocolate exposure) (Figure 6a,d).

In the NAc core, two-way ANOVA analysis of 1st chocolate exposure showed no significant difference of dialysate DA between groups (Figure 6b). In contrast, two-way ANOVA analysis of 2nd chocolate exposure showed a main effect of treatment ($F_{(1,10)}$= 12.64, $P<0.05$) and a significant time x treatment interaction ($F_{(12,120)}$=3.39, $P<0.05$). Bonferroni’s *post hoc* tests revealed an increase of dialysate DA in the NAc core of JWH-018 treated group after the 2nd chocolate exposure as compared to basal values (10’) and to Veh- treated group (90’) (Figure 6e). Then, data were analysed separately by Friedman’s test that revealed significant differences in the JWH-018 treated groups after the 2nd chocolate exposure (F_R=27.55, df 13,6, $P<0.05$). Dunn’s test revealed an increase of dialysate DA with respect to basal values (30’,40’, 80’, 90’) (Figure 6e).

Finally, in the mPFC, two-way ANOVA analysis of 1st chocolate exposure showed a main effect of time ($F_{(12,120)}$=10.43, $P<0.05$) and a significant time x treatment interaction ($F_{(12,120)}$=3.81, $P<0.05$). Bonferroni’s *post hoc* test revealed an increase of dialysate DA in the mPFC of JWH-018 treated group after the 1st chocolate exposure as compared to basal values (30’) (Figure 6c). Two-way ANOVA analysis of 2nd chocolate exposure showed a main effect of time ($F_{(12,120)}$=4.51, $P<0.05$), of treatment ($F_{(1,10)}$= 21.60, $P<0.05$) and a significant time x treatment interaction ($F_{(12,120)}$=2.64, $P<0.05$). Bonferroni’s *post hoc* tests revealed an increase of dialysate DA in the mPFC of Veh- treated group as compared to basal values and to JWH-018 treated group (20’-30’) (Figure 6f). Then, data were analysed separately by Friedman’s test that revealed significant differences in Veh-treated group after the 1st chocolate exposure (F_R=23.95, df 13,6, $P<0.05$) and the 2nd chocolate exposure (F_R=41.97, df 13,6, $P<0.05$), and in the JWH-018 treated groups after the 1st chocolate exposure (F_R=47.13, df 13,6, $P<0.05$).

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Dunn's test revealed an increase of dialysate DA with respect to basal values in the mPFC of both Veh-treated (10', 1st chocolate exposure; 10'-30', 2nd chocolate exposure) and JWH-018 treated groups (20'-30', 2nd chocolate exposure) (Figure 6c,f). During microdialysis experiments, taste reactions to chocolate exposures were also scored. Mann-Whitney test showed a significant increase of aversive score to the 1st chocolate exposure in JWH-018 treated as compared to Veh-treated group ($U=34.50$ $P<0.05$; Figure 6h), while no significant differences were observed during the 2nd chocolate exposure (Figure 6j). No differences in appetitive score during both chocolate exposures were observed (Figure 6g,i).

Effect of JWH-018 repeated exposure on GFAP and IBA-1 IR in mesocortical and mesolimbic DA areas either 24h or 7 days after JWH-018 discontinuation

In order to disclose whether JWH-018 repeated exposure was able to induce changes on glial cells (astrocytes and microglia), we evaluated 24h and 7 days after JWH-018 discontinuation the expression of GFAP and IBA-1 IR in mesocortical and mesolimbic DA areas.

Repeated JWH-018 exposure induces changes of GFAP and IBA-1 IR in the mPFC, NAc shell and core, and VTA both 24h and 7 days after JWH-018 discontinuation. Twenty-four hours after JWH-018 discontinuation, two-way ANOVA of GFAP-IR showed a main effect of treatment ($F_{(1,32)}=115.6$, $P<0.05$), of brain area ($F_{(3,32)}=37.95$, $P<0.05$), but not brain area × treatment interaction. To better evaluate the effect in each brain area, data were analysed separately. Student's t-test showed a significant increase of GFAP-IR levels in the mPFC (+23%; $t_{(8)}=4.82$, $P<0.05$), in the NAc shell (+42%; $t_{(8)}=5.04$, $P<0.05$) and core (+44%; $t_{(8)}=7.79$, $P<0.05$), and in the VTA (+24%; $t_{(8)}=4.69$, $P<0.05$) of JWH-018-treated as compared to Veh-treated group (Figure 7a). Considering IBA-1 expression, two-way ANOVA showed a main effect of treatment ($F_{(1,32)}=41.40$, $P<0.05$; $P<0.05$) and brain area ($F_{(3,32)}=14.23$, $P<0.05$) but not a brain area × treatment significant interaction. Subsequent analysis by Student's t-tests showed a significant increase of IBA-1 positive cells in the NAc

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shell (+17%; $t_{(8)}$=3.67, P<0.05) and core (+28%; $t_{(8)}$=6.18, P<0.05), and in the VTA (+20%; $t_{(8)}$=2.31, P<0.05) of JWH-018-treated rats as compared to Veh-treated group (Figure 7b). Similarly, 7 days after JWH-018 discontinuation, two-way ANOVA of GFAP-IR showed a main effect of treatment ($F_{(1,32)}$= 42.60 , P<0.05) and brain area ($F_{(3,32)}$=5.45, P<0.05) but not a brain area × treatment significant interaction. Student's t-tests revealed a significant increase of GFAP-IR levels in the mPFC (+37%; $t_{(8)}$=3.01, P<0.05), NAc shell (+42%; $t_{(8)}$=3.00, P<0.05) and core (+32%; $t_{(8)}$=3.36, P<0.05) and in the VTA (+27%; $t_{(8)}$=7.38, P<0.05) of JWH-018-treated as compared to Veh-treated group (Figure 8a). Considering IBA-1 IR, two-way ANOVA showed a main effect of treatment ($F_{(1,32)}$=19.49, P<0.05), of brain area ($F_{(3,32)}$=11.68, P<0.05) but not brain area × treatment significant interaction. Student's t-tests revealed a significant increase of IBA-1 positive cells in the NAc shell (+30%; $t_{(8)}$=3.15, P<0.05) and core (+31%; $t_{(8)}$=3.03, P<0.05) of JWH-018-treated as compared to Veh-treated group (Figure 8b).

Effect of JWH-018 repeated exposure on CB1Rs expression in mPFC, NAc shell and core 7 days after JWH-018 discontinuation

Finally, to assess whether JWH-018 repeated exposure was able to induce persistent changes in CB1Rs, we evaluated the expression of CB1Rs in mPFC and NAc shell and core 7 days after JWH-018 discontinuation. Two-way ANOVA showed a main effect of treatment ($F_{(1,24)}$=44.59, P<0.05), of brain area ($F_{(2,24)}$= 73.38, P<0.05) but not brain area × treatment interaction. To better evaluate the effect in each brain area, data were analysed separately. Student's t-tests revealed a significant decrease of CB1R-IR in the mPFC (-23%; $t_{(8)}$=2.66, P<0.05), NAc shell (-29%; $t_{(8)}$=5.63, P<0.05, p=0.0005) and core (-42%; $t_{(8)}$=8.07, P<0.05) of JWH-018-treated as compared to Veh-treated group (Figure 9).

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For all immunostaining (TH-IR, GFAP-IR, IBA-1 IR, CB1R-IR), standard control experiments were performed by omitting either the primary or secondary antibodies and yielded no cellular labelling (data not shown).

DISCUSSION

The main findings of this study are threefold. First, repeated exposure to JWH-018 and subsequent discontinuation induces behavioural, electrophysiological and neurochemical changes related to withdrawal. Second, this treatment induces differential adaptive changes on dialysate DA in the NAc shell and core, and in the mPFC in response to a single or recurrent presentation of an appetitive salient stimulus. Finally, these changes are associated with astrogliosis, microgliosis and downregulation of CB1Rs in selected mesolimbic and mesocortical DA brain areas.

Repeated JWH-018 exposure induced an anxiety-like phenotype, as revealed by the reduction of time spent in the open arms of the EPM, at 1h and 24h, but not 7 days, after drug discontinuation. Notably, JWH-018- and vehicle-treated rats displayed no differences in the total number of entries in the arms of the EPM, which support the view that reduction of the time spent in the open arms by JWH-018-treated rats stems from the presence of an anxiety-like state rather than from non-specific drug effects on arm exploration (i.e. alteration of locomotor activity). Notably, JWH-018-treated rats displayed a similar reduction in the time spent in the open arms 24h after JWH-018 administration, thus demonstrating that the modifications in the anxiety-like states induced by repeated JWH-018 begin during treatment and persist after drug discontinuation. Results of the MB test provide additional support to the possibility that repeated exposure to JWH-018 has anxiogenic effects. Indeed, JWH-018-treated rats showed increased burying scores, which are compatible with the presence of an anxiety-like state (Jimenez-Gomez, Osentoski, & Woods, 2011) and/or compulsive-repetitive behaviours (Zanda et al., 2017). Taken together, these behavioural abnormalities might be

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due to a dysregulation of eCB system, which is known to modulate anxiety-related responses (Macri et al., 2013; Parolaro, Realini, Vigano, Guidali, & Rubino, 2010). Consistently, in humans after regular marijuana use (Sideli, Quigley, La Cascia, & Murray, 2020; Volkow et al., 2017) and in animals after repeated administration of SCRAs, altered anxiety-related responses have been observed (Macri et al., 2013). Moreover, use of SCRAs has been associated with acute (Papanti et al., 2014) and persistent psychosis (Sideli et al., 2020). Importantly, we found that in the PPI test repeated exposure to JWH-018 induced transitory reductions of sensorimotor-gating at 1h after the last JWH-018 administration. Few clinical and preclinical studies have investigated the impact of cannabis consumption on PPI and reported mixed results (Quednow, Kuhn, Hoenig, Maier, & Wagner, 2004; Tournier & Ginovart, 2014). Our findings are in line with the notion that SC may possess psychotomimetic potential (Deng, Verrico, Kosten, & Nielsen, 2018; Fattore, 2016). Finally, in an attempt to get a better picture of behavioural anomalies relevant to hedonic/aversive state, we evaluated potential modifications of taste reactions to an intraoral sweet chocolate solution. We found that the 1st (but not the 2nd) exposure to chocolate elicited significantly higher aversive taste reactions in JWH-018-treated than Veh-treated rats, which suggests taste neophobia and confirms an aversive state in rats treated with JWH-018. Moreover, similarly to findings described in mice after subcutaneous THC (1-50 mg/kg) or JWH-018 (1 mg/kg) treatment (Trexler et al., 2018), we observed marked spontaneous somatic signs of withdrawal in JWH-018-treated rats at 24h and, to a lesser extent, 7 days after JWH-018 discontinuation.

Electrophysiological recording of DA neurons located in the parabrachial pigmented nuclei of posterior VTA, a subregion densely populated of DA cells projecting to NAc (Yamaguchi, Wang, Li, Ng, & Morales, 2011), showed that repeated JWH-018 exposure induces changes in basal DA neuron activity. In particular, we observed a decreased number

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of both spontaneously active VTA DA and TH-positive cells both 24h and 7 days after JWH-018 discontinuation and a decrease in firing rate at 7 days. These observations indicate a hypodopaminergic state (Melis, Spiga, & Diana, 2005) during withdrawal from JWH-018, consistent with previous observations reported after chronic exposure to other drugs of abuse (Cannizzaro et al., 2019; Diana et al., 2003; Diana et al., 1998; Diana, Muntoni, Pistis, Melis, & Gessa, 1999). The mechanism responsible for changes in VTA DA cell activity is not clear yet. One possibility is that repeated JWH-018 administration induces a profound and long-lasting downregulation of CB1Rs expressed on GABA or Glu afferents to DA neurons, as consistently shown in other brain regions such as the hippocampus (Dudok et al., 2015). This change in CB1R activity might disrupt the balance between excitatory and inhibitory afferents impinging on DA cells, leading to a decrease in spontaneous activity. Consistently, we observed a decrease of CB1Rs expression in the mPFC, (also in the NAc shell and core), which exerts an important regulatory action on DA signalling through its projection (Carr & Sesack, 2000; Taber, Das, & Fibiger, 1995). Nevertheless our findings warrant further studies in order to recognise the projections of the single neurons recorded, as well as to evaluate the CB1Rs expression in specific cell types (i.e. neurons, astrocytes). Notably, these changes do not reflect differences between groups in DA extracellular basal levels in NAc shell or core neither at 24h nor at 7 days after drug discontinuation. Yet, reduced DA basal levels have been detected at 24h in the mPFC of JWH-018-treated as compared to Veh-treated animals, suggesting an alteration of mPFC DA transmission during acute withdrawal that may account for lack of its inhibitory control on the subcortical areas examined (i.e. NAc shell and core). This specific aspect has been studied in more depth by using a salient stimulus (i.e. intraoral chocolate) with a putative motivational value.

The microdialysis experiments showed that repeated JWH-018 exposure reduces and potentiates the stimulation of DA transmission in the NAc shell and core, respectively, in

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response to acute JWH-018 challenge. This differential adaptation of NAc shell and core DA resembles previous observations of reciprocal changes in the responsiveness of NAc shell/core DA to a THC challenge in rats sensitised to increasing doses of THC (Cadoni, Valentini, & Di Chiara, 2008). After 7 days of withdrawal from JWH-018, we also observed differential changes in the response of NAc shell and core and mPFC DA to an unfamiliar appetitive taste stimulus. On the first chocolate exposure, Veh-treated rats showed an increase of dialysate DA in the NAc shell and in the mPFC but not in the NAc core. In rats withdrawing from chronic JWH-018, however, dialysate DA still increased in the mPFC but not in the NAc shell and core. On a second chocolate exposure, dialysate DA increased in the mPFC, but not in the NAc shell and core, of Veh-treated rats. In contrast, in rats withdrawing from JWH-018, dialysate DA fully increased in the NAc shell and core but not in the mPFC. Thus, after pre-exposure to chocolate, rats repeatedly treated with JWH-018, in contrast to Veh-treated animals (present findings) and naive animals (Bassareo et al., 2002), showed downregulation of DA responsiveness in the mPFC and a stimulatory DA response in the NAc shell and core. This reciprocal effect of DA responsiveness in the mPFC and in the NAc subdivisions could be due to the relief of DA transmission in the NAc from the inhibitory influence exerted by mPFC DA, althought further experiments would be necessary to confirm this possibility. These findings would imply that repeated JWH-018 exposure, at variance with morphine sensitisation (De Luca et al., 2011) and ablation of DA in the mPFC (Bimpisidis et al., 2013), decreases the sensitivity of the NAc shell to the first exposure to a natural rewarding stimulus like chocolate. This could be due to the influence of the aversive state associated to JWH-018 withdrawal, as shown by the behavioural results. This interpretation is in line with the hypothesis that DA codes for novelty and motivational valence of aversive and hedonic stimuli (Bassareo et al., 2002).

Changes in neural-glial interactions play a role in the development and maintenance

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of drug dependence (Lacagnina et al., 2017) and chronic use of addictive drugs leads to glial alterations (Cutando et al., 2013; Kim, Healey, Sepulveda-Orengo, & Reissner, 2018). In this study, 24h after the last JWH-018 administration, we observed astrogliosis and microgliosis in different DA brain areas, which persisted up to 7 days after JWH-018 discontinuation. In particular, increased expression of GFAP has been observed in all brain areas examined within the mesocorticolimbic DA pathways (mPFC, NAc shell/core, VTA), together with an increased number of IBA-1 positive cells in the NAc shell and core, and in the VTA. Although we did not perform a stereological cell counting, the total size of the examined area in which IBA-1 and TH neurons, as well GFAP fibers were counted, was chosen according to the Rat Brain Atlas coordinates (Paxinos & Watson, 2007) in order to include almost the whole area of each region analysed. Modifications in astrocyte structure and function have been associated to neuropsychiatric diseases, including addiction (Kim et al., 2018), resulting in either astrogliosis or astrocytopathy depending on drug class, dose, route of administration, length of withdrawal and brain areas examined (Castelli et al., 2014; Fattore et al., 2002; Miguel-Hidalgo, 2009). Besides astrogliosis, our results revealed an increased number of microglia cells in the NAc shell and core, and in the VTA of JWH-018-treated rats, confirming the involvement of both types of glial cells in the effects of drugs of abuse (Lacagnina et al., 2017; Melis et al., 2017). In addition to changes in glial cells, we observed a concomitant CB1Rs downregulation as previously observed in mice after repeated THC exposure (Cutando et al., 2013; Zamberletti, Gabaglio, Prini, Rubino, & Parolaro, 2015), supporting the relationship between the eCB system function in glial cell physiology and neuroinflammation. Notably, the persistence of these glial alterations up to 7 days after JWH-018 discontinuation is in line with observations with other drugs of abuse, i.e. cocaine and amphetamines (Bowers & Kalivas, 2003; Granado et al., 2011). Interestingly, several parallel studies reported a reduction in astrocyte glutamate transporter-1 (GLT-1) function during

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cocaine, ethanol, or morphine withdrawal (Das, Althobaiti, Alshehri, & Sari, 2016; Fischer-Smith, Houston, & Rebec, 2012; Kim et al., 2018), supporting the role of astrocytes in Glu homeostasis at neuronal synapses (Bjornsen, Hadera, Zhou, Danbolt, & Sonnewald, 2014), in line with the concept of the "tripartite" (Hammond, Cayre, Panatier, & Avignone, 2015), now evolved in "quadripartite", synapse (Fouyssac & Belin, 2019). Consistently, we observed that Glu and GABA extracellular levels were markedly reduced in the mPFC 24h after the last JWH-018 administration, most likely due to a modified expression and/or activity of GABA and Glu glial transporters. In light of these results, repeated JWH-018 exposure might likely induce changes in eCB signalling (e.g., CB1Rs downregulation in DA terminal areas), which through modifications in glial cell expression and activity might induce a perturbation of Glu signalling and alter the balance between GABA and Glu systems. This, in turn, might partially contribute to behavioural anomalies and dysregulation of the mesolimbic and mesocortical DA systems. Indeed, recent findings have demonstrated profound disturbances of the mesocorticolimbic circuitry following adolescent (but not adult) THC exposure, characterised by long-lasting GABA hypoactivity and Glu hyperactivity in the PFC, a sub-cortical hyper-DA neuronal phenotype in the VTA, impairments in short-term memory, social motivation and cognition, increased anxiety levels and decreased motivation (Renard, Rosen, et al., 2017; Renard, Szkudlarek, et al., 2017). These discrepancies with our results may be due to differences in the drug tested (natural vs synthetic cannabinoid), in the evaluation time intervals (7 vs 30 days of drug withdrawal) and/or in the lifetime period of drug exposure (adolescence vs adulthood) . In particular, the latter point may represent a discriminative factor in the different consequences observed, since adolescence is a critical neurodevelopmental period characterised by increased neural plasticity and vulnerability to chemical insult (such as cannabinoids exposure), particularly in cortical and limbic regions (Hurd, Michaelides, Miller, & Jutras-Aswad, 2014; Spear, 2000).

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Taken together, the results obtained in rats repeatedly exposed to JWH-018 recapitulate some typical aspects of drug dependence, including the induction of an aversive state upon withdrawal and loss of responsiveness of NAc shell and core DA to a novel palatable reinforcer. Reinstatement of NAc DA response by a second exposure to the palatable taste suggests that a taste reward can overcome the inhibitory influence of JWH-018 withdrawal on NAc DA transmission. Finally, the changes of glial cells in DA brain areas support the hypothesis of a role of neuroinflammation in cannabinoid dependence. In light of evidence showing sex differences in cannabinoid addiction (Fattore et al., 2007), DA neurotransmission (Becker, 2016), and neuroinflammation (Liu, Li, Su, Wang, & Jiang, 2019), future studies will assess JWH-induced effects in male and female rats by using the self-administration paradigm to better mimic human exposure pattern of drug intake.

AUTHOR CONTRIBUTIONS

M.A.D.L. and G.D.C. designed the study. N.P., M.P.C., G.D.C., and M.A.D.L. wrote the manuscript. N.P., C.M., and G.F., planned, performed and analysed microdyalisis experiments, C.S., M.D.F., and M.P., planned, performed and analysed electrophysiological experiments, N.S., N.P., M.S., L.F., and P.F., planned, performed and analysed behavioural experiments; N.P., M.P.C., and M.G.E., planned, performed and analysed immunohistochemical experiments. All authors have read and agreed to the final version of the manuscript.

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, Immunoblotting and Immunochemistry, and Animal Experimentation,

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and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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XII. FIGURES AND TABLES

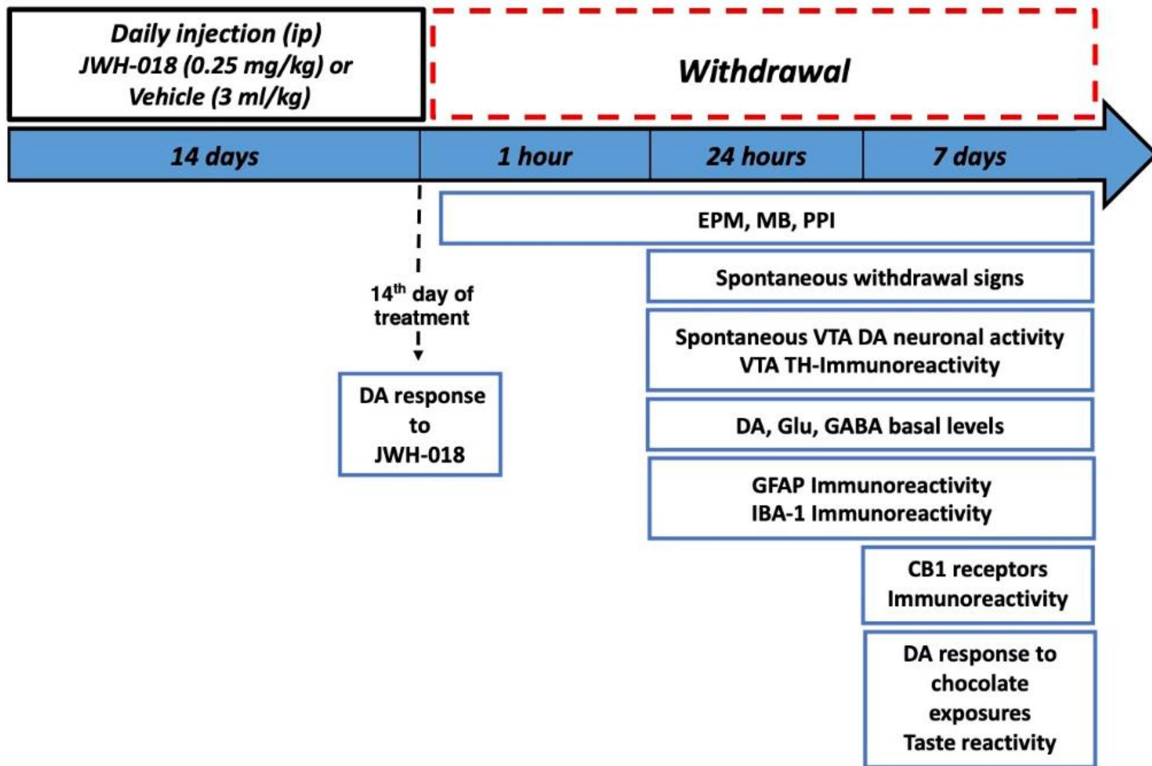

Figure 1. Time schedule of JWH-018 exposure and experimental design. DA, Dopamine; CB, cannabinoid; EPM, Elevated Plus Maze; GFAP, Glial Fibrillary Acidic Protein; Glu, Glutamate; IBA-1, Ionized calcium Binding Adaptor molecule-1; MB, Marble Burying test; PPI, Prepulse Inhibition; TH, Tyrosine Hydroxylase; VTA, Ventral Tegmental Area.

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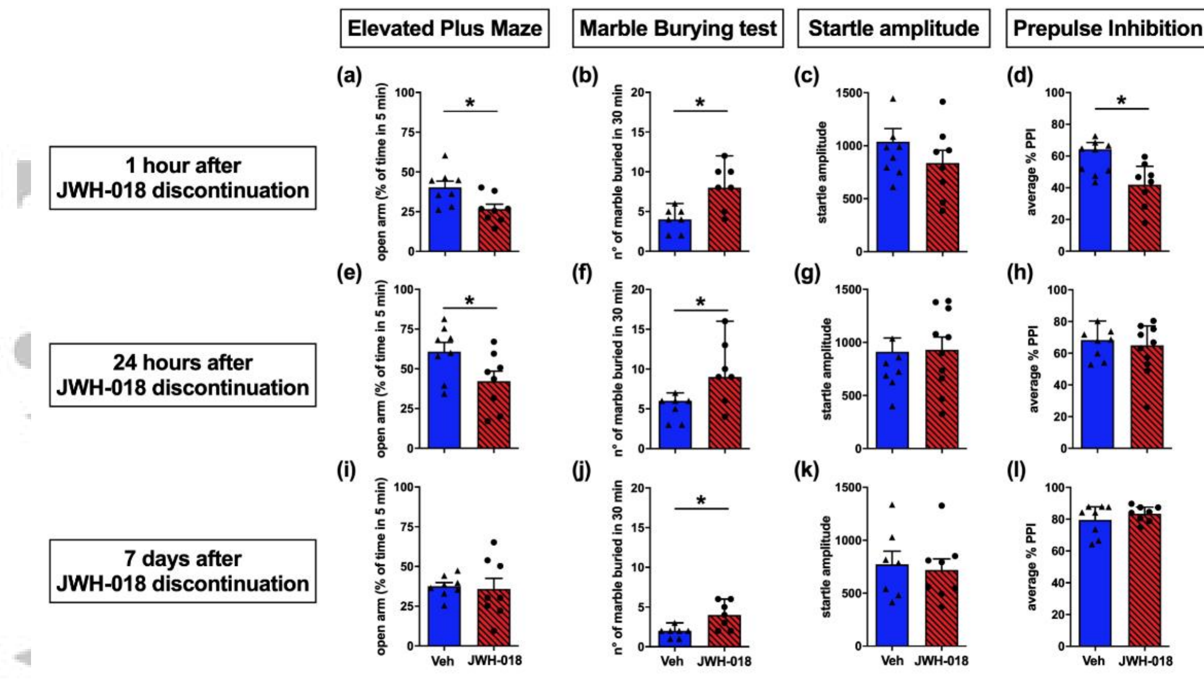

Figure 2. Repeated JWH-018 exposure induces time-dependent abnormalities in EPM, MB, PPI. Bars represent mean±SEM of the percentage of time spent in open arms during the EPM test (Veh n=8, JWH-018 n=8, unpaired Student's t-tests, panels a,e,i); median±95% CI of the total number of marbles covered with bedding during the MB test (Veh n=7, JWH-018 n=7, Mann-Withney U-test, panels b,f,j), mean±SEM of startle amplitude (panels c,g,k) and median±95% CI of average of the percentage of PPI (1h, Veh: n=9, JWH-018: n=8, panel d; 24h, Veh: n=8, JWH-018: n=11, h; 7 days, Veh n=8, JWH-018 n=8, Mann-Withney U-test, panel l) in Vehicle- (*blue bars*) and JWH-018- (*red bars*) exposed rats at 1h, 24h and 7 days after drug discontinuation. *$p<0.05$ vs Veh.

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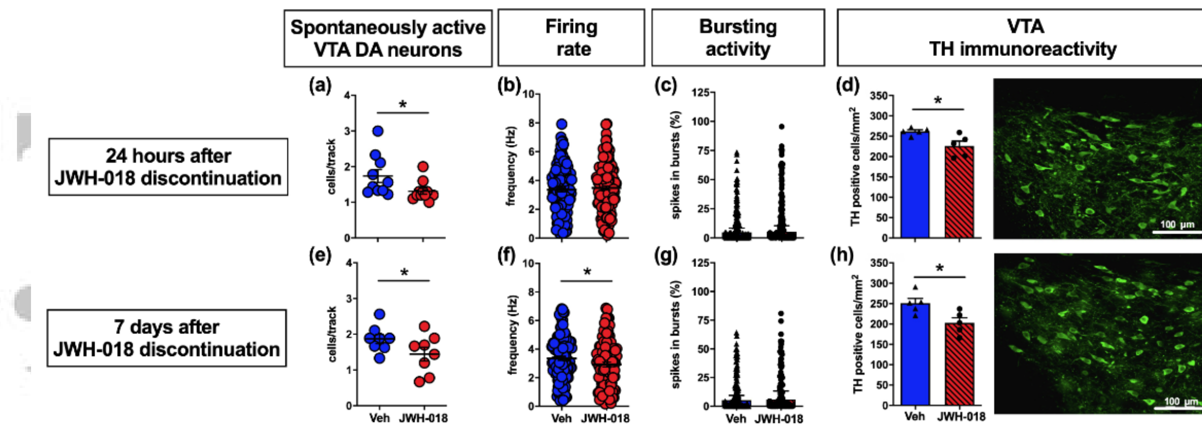

Figure 3. Repeated JWH-018 exposure induces persistent modifications in VTA DA neurons activity. Data are presented as mean±SEM of the number of spontaneously active VTA DA neurons (24h, Veh: n=10; JWHn=11, panel a; 7 days, Veh: n=8; JWH: n=8, unpaired Student's t-tests, panel e) and firing rate of VTA DA neurons (24h, Veh: n=144 from 10 rats; JWH: n=132 from 11 rats, panel b; 7 days, Veh: n=125 from 8 rats; JWH: n=96 from 8 rats, unpaired Student's t-tests, panel f) recorded from Veh- (*blue circles*) and JWH-018- (*red circles*) exposed rats at 24h and 7 days after drug discontinuation. The horizontal lines represent average values that are significantly different between the 2 groups. Bars represent median±95% CI of the percentage of spikes in bursts (24h, Veh: n=144 from 10 rats; JWH: n=132 from 11 rats, panel c; 7 days, Veh: n=125 from 8 rats; JWH: n=96 from 8 rats, Mann-Whitney U-test, panel g), and mean±SEM of number of TH positive cells in the VTA expressed per mm^2 in Veh- (*blue bars*) and JWH-018- (*red bars*) exposed rats at 24h and 7 days after drug discontinuation (Veh n=5, JWH-018 n=5, unpaired Student's t-tests, panels d,h). Representative images of TH expression in Vehicle (top) and JWH-018 treated rats (bottom). *$p<0.05$ vs Veh.

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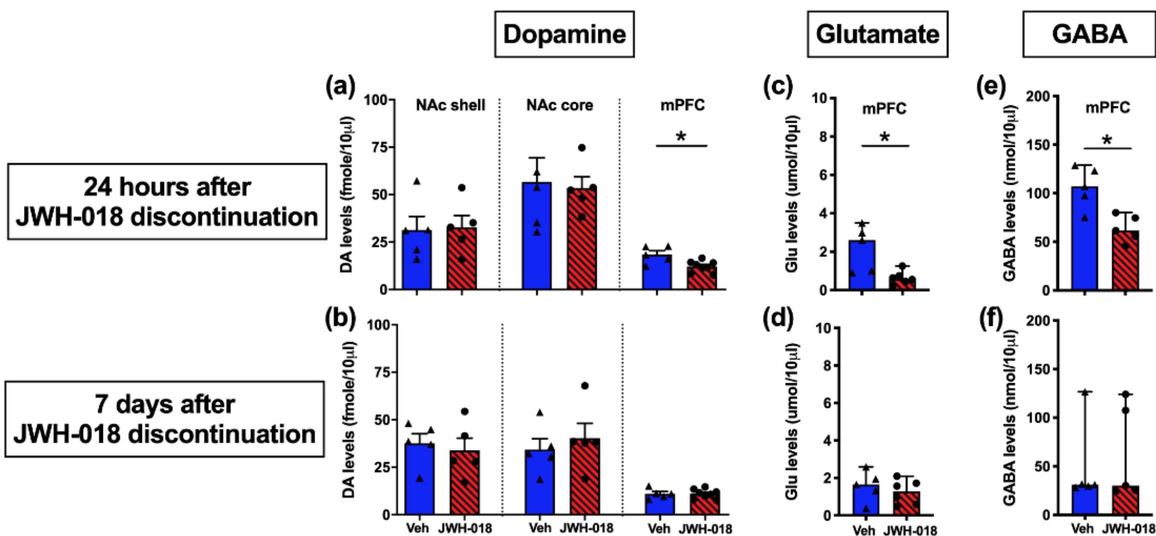

Figure 4. Effect of JWH-018 repeated exposure on extracellular basal DA, Glu, and GABA levels in the NAc and mPFC, at 24h and 7 days after drug discontinuation. Bars represent mean ± SEM of basal extracellular levels of DA expressed as fmoles/10μl (panels a,b), and median±95% CI of Glu (panels c,d) and GABA (panels e,f) expressed as μmoles/10μl and nmoles/10μl sample, respectively. The bars represent the basal DA (NAc shell: Veh n=5, JWH-018 n=5; NAc core: Veh n=5, JWH-018 n=5; mPFC: Veh n=5, JWH-018 n=8, unpaired Student's t-test), basal Glu (Veh n=5, JWH-018 n=6, Mann-Withney U-test) and basal GABA (Veh n=5, JWH-018 n=5, Mann-Withney U-test) levels 24h and 7 days after JWH-018 discontinuation. $*p<0.05$ vs Veh.

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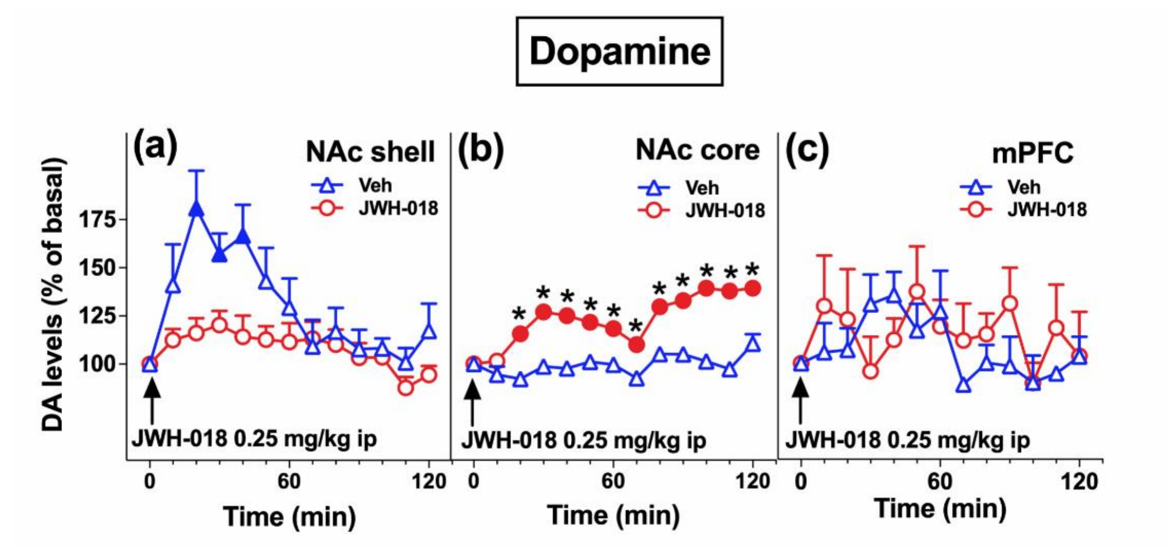

Figure 5. Repeated JWH-018 exposure induces differential adaptive changes on the responsiveness of NAc shell and core DA, but not of mPFC DA to JWH-018 injection during the last day of treatment. Data are presented as mean±SEM of change in DA extracellular levels expressed as the percentage of basal values. The arrow indicates the start of JWH-018 ip injection (0.25mg/kg) in Veh- (*blue triangles*) and JWH-018- (*red circles*) exposed rats implanted in the NAc shell (Veh: n=7; JWH-018: n=6; panel a), NAc core (Veh: n=6; JWH-018: n=6; panel b), and in the mPFC (Veh: n=5; JWH-018: n=5; panel c). Solid symbols: $p<0.05$ with respect to basal values; *$p<0.05$ vs Veh (RM Two-way ANOVA followed by Bonferroni's *post hoc*; Friedman's test followed by Dunn's *post hoc*).

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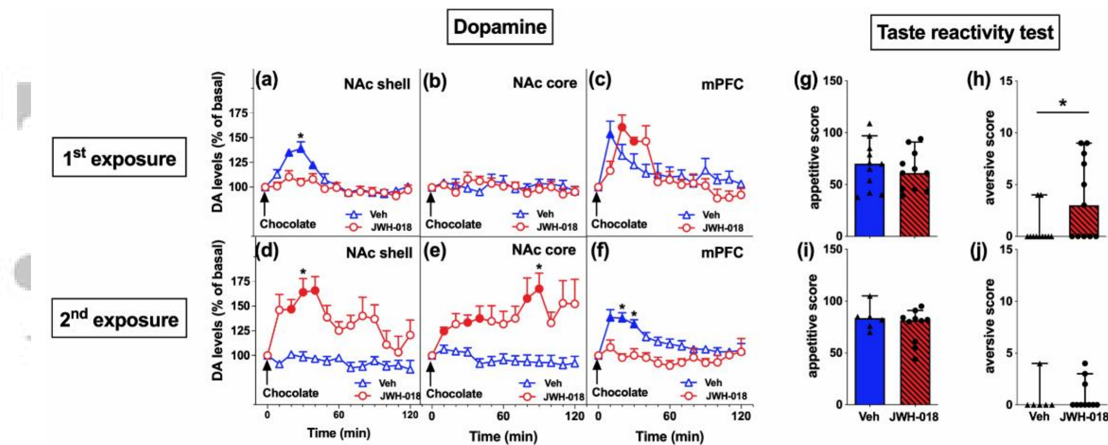

Figure 6. Repeated JWH-018 exposure induces differential adaptive changes on the responsiveness of NAc shell, NAc core, and mPFC DA, and changes on taste reactions to repeated chocolate exposure evaluated 7 days after JWH-018 discontinuation. Data are presented as mean±SEM of change in DA extracellular levels expressed as the percentage of basal values and as median±95% CI of behavioural appetitive and aversive score to chocolate. The arrow indicates the start of chocolate intraoral infusion (1ml/5min) in Veh- (*blue triangles*) and JWH-018- (*red circles*) exposed rats implanted in the NAc shell (Veh: n=6; JWH-018: n=6; panel a,d), NAc core (Veh: n=6; JWH-018: n=6; panel b,e), and in the mPFC (Veh: n=6; JWH-018: n=6; panel c,f). Solid symbols: $p<0.05$ with respect to basal values; *$p<0.05$ Veh- vs JWH-018 within the 1st or 2nd chocolate exposure group (RM Two-way ANOVA followed by Bonferroni's *post hoc*; Friedman's test followed by Dunn's *post hoc).* The bars represent appetitive (1st exp, Veh: n=11; JWH-018: n=11; panel g; 2nd exp, Veh: n=6, JWH-018: n= 10, panel i) and aversive (1st exp, Veh: n=11; JWH-018: n=11; panel h; 2nd exp, Veh: n=6, JWH-018: n=10, panel j) taste reactions to the 1st and the 2nd chocolate exposure. *$p<0.05$ vs Veh (Mann-Whitney test).

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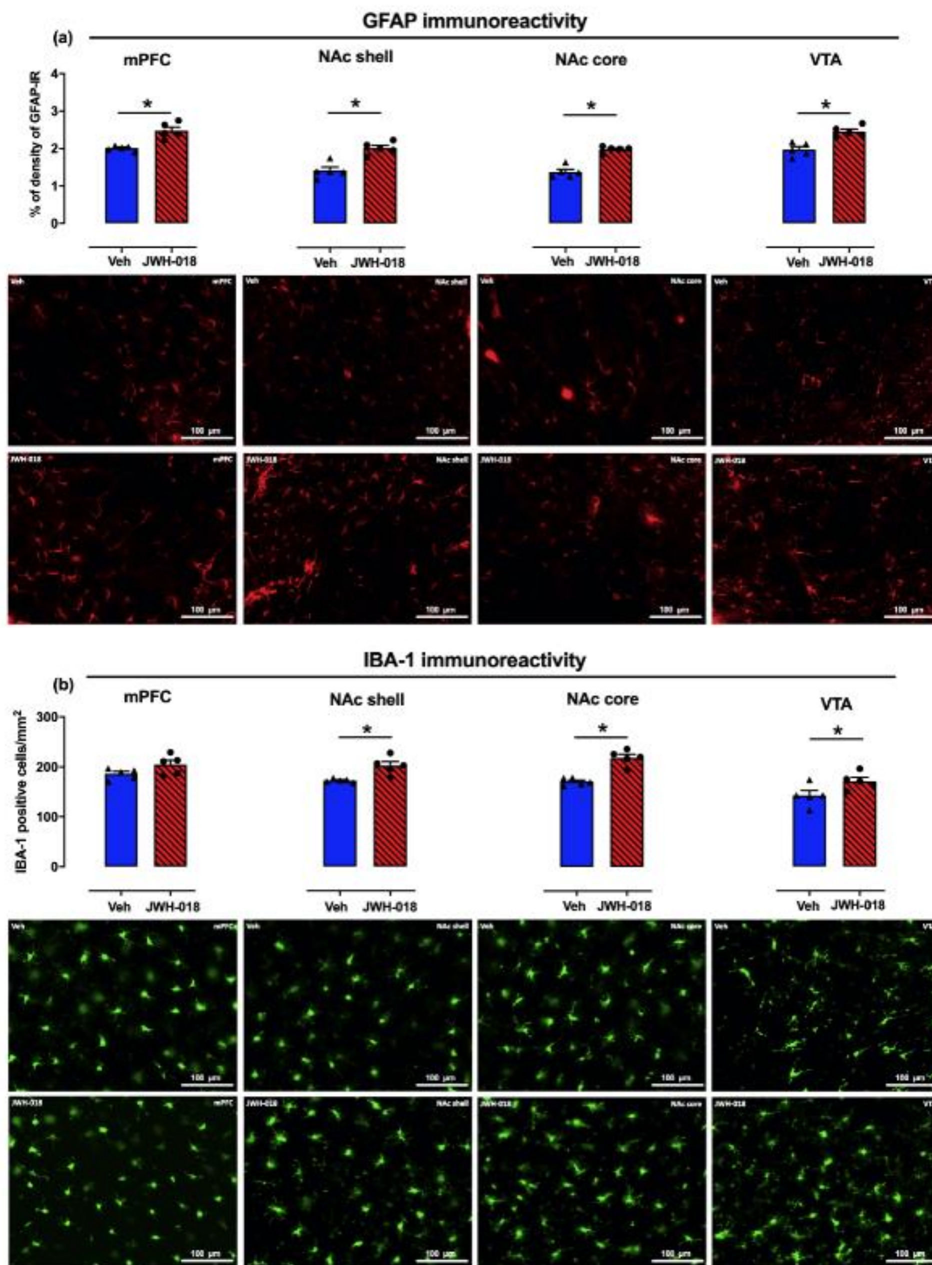

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Figure 7. Repeated JWH-018 exposure induces changes on GFAP and IBA-1 immunoreactivity in mPFC, NAc shell and core and VTA 24h after JWH-018 discontinuation. Data are presented as mean±SEM of density reading expressed as a percentage of the area covered by GFAP-IR and of the number of IBA-1 positive cells, expressed per mm^2. The bars represent the percentage of area covered in different DA brain regions by GFAP-IR (Veh: n=5; JWH-018: n=5; panel a) and the number of IBA-1 positive cells (Veh: n=5; JWH-018: n=5; panel b). Representative images of GFAP and IBA-1 expression in Veh- (*top*) and JWH-018-treated rats (*bottom*), in the mPFC, NAc (shell and core) and VTA, respectively. *$p<0.05$ vs Veh (unpaired Student's t-tests).

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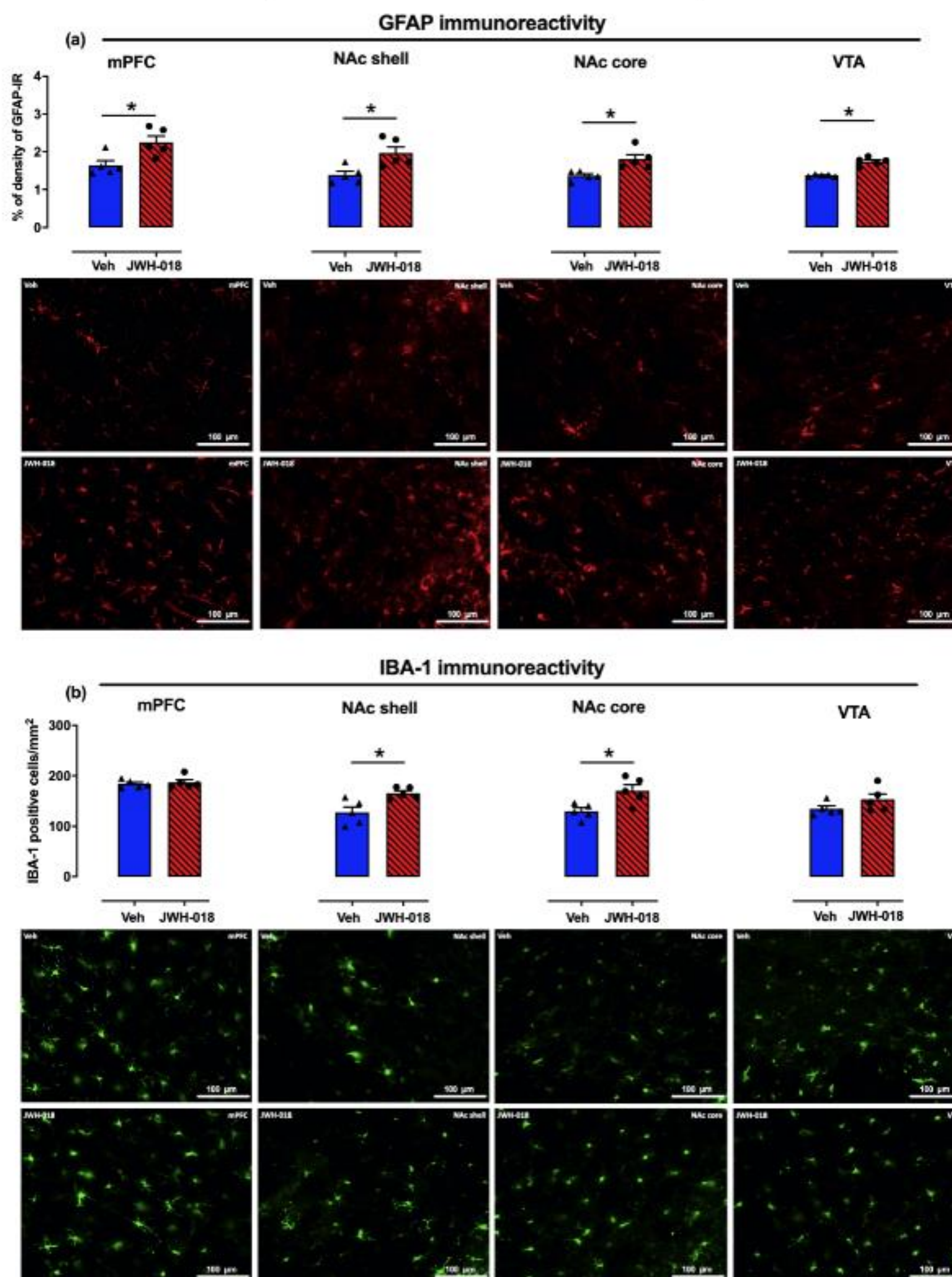

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Figure 8. Repeated JWH-018 exposure induces changes on GFAP and IBA-1 immunoreactivity in mPFC, NAc shell and core, and VTA 7 days after JWH-018 discontinuation. Data are presented as mean±SEM of density reading expressed as a percentage of the area covered by GFAP-IR and of the number of IBA-1 positive cells, expressed per mm^2. The bars represent the percentage of area covered in different DA brain regions by GFAP-IR (Veh: n=5; JWH-018: n=5; panel a) and the number of IBA-1 positive cells (Veh: n=5; JWH-018: n=5; panel b). Representative images of GFAP and IBA-1 expression in Veh- (*top*) and JWH-018- treated rats (*bottom*), in the mPFC, NAc (shell and core) and VTA, respectively. *$p<0.05$ vs Veh (unpaired Student's t-tests).

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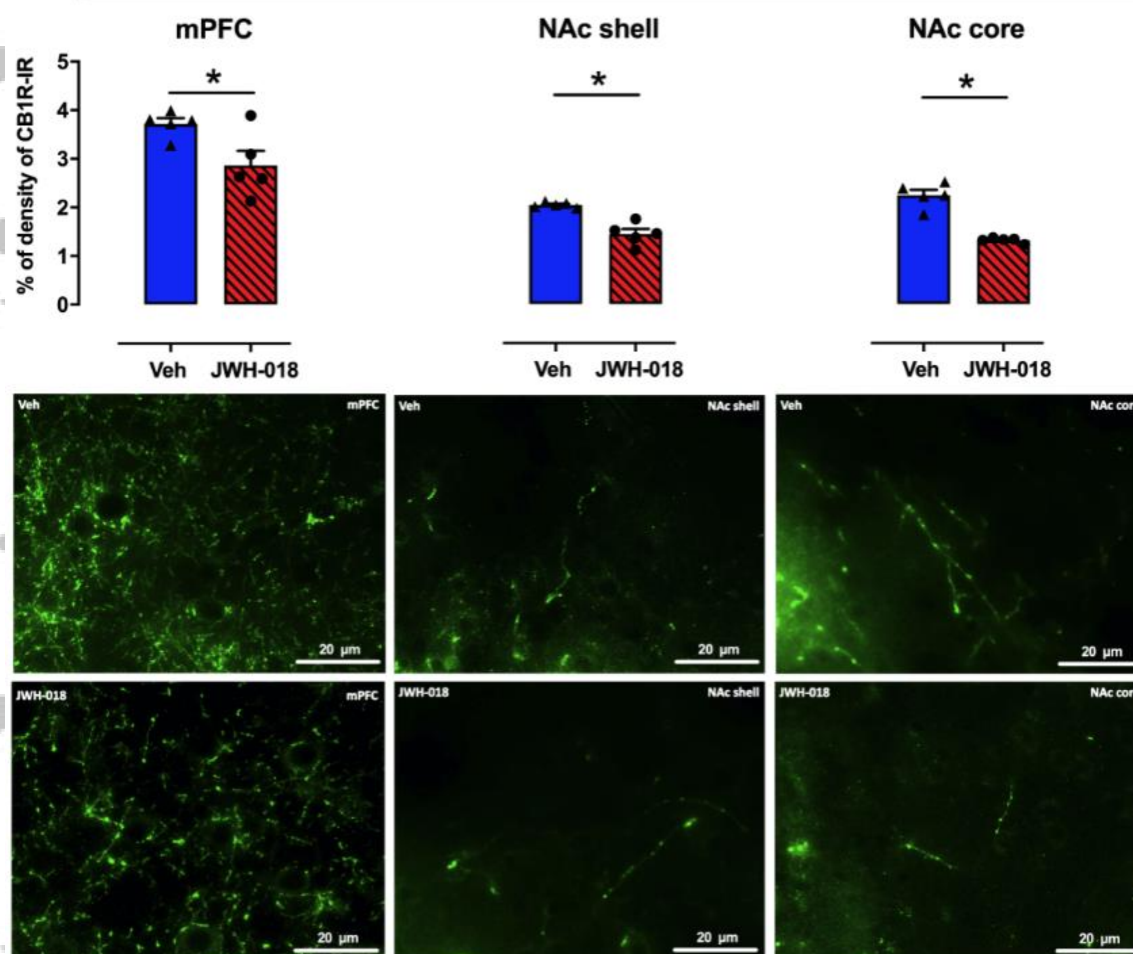

Figure 9. Repeated JWH-018 exposure induces changes on CB1Rs expression in mPFC, NAc shell and core 7 days after JWH-018 discontinuation. Data are presented as mean±SEM of density reading expressed as a percentage of the area covered by CB1R-IR. The bars represent the percentage of area covered by CB1R-IR in the mPFC, in NAc shell and core (Veh: n=5; JWH-018: n=5). Representative images of CB1R expression in Veh- (*top*) and JWH-018 treated rats (*bottom*) in the mPFC, NAc (shell and core). **p<0.05* vs Veh (unpaired Student's t-tests).

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Signs of withdrawal (30-min observation)	24 hours		7 days	
	Veh	JWH-018	Veh	JWH-018
facial rubbing	2 (0-5)	2 (1-3)	1 (0-3)	2 (1-3)
licking	1 (0-4)	4 (2-6)*	2 (0-5)	3 (1-7)
wet dog shakes	0	0 (0-1)	0	0
arched back	0	0 (0-1)	0 (0-4)	0
biting	0	1 (0-4)*	0	1 (0-2)*
head shakes	0	0 (0-1)*	0 (0-2)	0 (0-3)
chewing	0 (0-5)	5 (2-14)*	5 (1-9)	5 (1-19)
tongue rolling	0	1 (0-4)*	0 (0-1)	0 (0-2)
paw treading	0	0 (0-1)	0	0
forepaw fluttering	0 (0-1)	1 (0-1)	1 (0-4)	2 (0-3)
teeth chattering	0	1 (0-3)*	1 (0-4)	1 (0-1)
scratching	0.5 (0-1)	1 (0-2)	0 (0-1)	0 (0-1)

Table 1. Spontaneous somatic signs of withdrawal. Data are expressed as median±95% CI of behavioural withdrawal scores after a 30-min observation 24h (Veh: n=12; JWH-018: n=13) and 7 days (Veh: n=9; JWH-018: n=9) after JWH-018 discontinuation. *$p<0.05$ vs Veh (Mann-Whitney U-test).

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BULLET POINT SUMMARY

What is already known

- JWH-018 is a SCRA detected in “Spice/K2 drugs” marketed as legal marijuana surrogates
- The central effects induced by repeated exposure to SCRAs are underestimated

What this study adds

- Repeated JWH-018 exposure induces anxious and aversive behaviours, transitory attentional deficits and withdrawal signs
- Repeated JWH-018 exposure alters basal activity and responsiveness of DA transmission, while inducing glial and CB1Rs modification

What is the clinical significance

- Our data are useful to elucidate the severe consequences of recurring use of Spice/K2 drugs
- Our data might help clinicians to manage synthetic cannabinoid intoxications and related use disorders

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