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Immune and glial cell alterations in the rat brain after repeated exposure to the synthetic cannabinoid JWH-018

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ABSTRACT

The use of synthetic cannabinoid receptor agonists (SCRAs) poses major psychiatric risks. We previously showed that repeated exposure to the prototypical SCRA JWH-018 induces alterations in dopamine (DA) transmission, abnormalities in the emotional state, and glial cell activation in the mesocorticolimbic DA circuits of rats. Despite growing evidence suggesting the relationship between substance use disorders (SUD) and neuroinflammation, little is known about the impact of SCRAs on the neuroimmune system. Here, we investigated whether repeated JWH-018 exposure altered neuroimmune signaling, which could be linked with previously reported central effects.

Adult male Sprague-Dawley (SD) rats were exposed to JWH-018 (0.25 mg/kg, i.p.) for fourteen consecutive days, and the expression of cytokines, chemokines, and growth factors was measured seven days after treatment discontinuation in the striatum, cortex, and hippocampus. Moreover, microglial (ionized calcium-binding adaptor molecule 1, IBA-1) and astrocyte (glial fibrillary acidic protein, GFAP) activation markers were evaluated in the caudate-putamen (CPu).

Repeated JWH-018 exposure induces a perturbation of neuroimmune signaling specifically in the striatum, as shown by increased levels of cytokines [interleukins (IL) -2, -4, -12p70, -13, interferon (IFN) γ], chemokines [macrophage inflammatory protein (MIP) -1α, -3α], and growth factors [macrophage colony-stimulating factor (M-CSF), vascular endothelial growth factor (VEGF)], together with increased IBA-1 and GFAP expression in the CPu.

JWH-018 exposure induces persistant brain region-specific immune alterations up to seven days after drug discontinuation, which may contribute to the behavioral and neurochemical dysregulations in striatal areas that play a role in the reward-related processes that are frequently impaired in SUD.

1. Introduction

New psychoactive substances (NPS) designed to mimic the psychoactive effects of illicit drugs of abuse have emerged as an urgent public health concern (UNODC, 2022). Current NPS classes include SCRAs, stimulants, opioids, benzodiazepines (and other sedative-hypnotics), hallucinogens, and dissociatives. Since 2008, more than 200 SCRAs have been detected in the illegal drug market including 15 first described in 2021, representing the largest group of substances being monitored by the European Monitoring Centre for Drugs and Drug

Abbreviations: BSA, bovine serum albumin; CBR, cannabinoid receptor; CPu, Caudate-Putamen; DA, dopamine; EMCDDA, European Monitoring Centre for Drugs and Drug Addiction; G-CSF, granulocyte colony-stimulating factor; GFAP, glial fibrillary acidic protein; GM-CSF, granulocyte macrophage colony-stimulating factor; GRO/KC, keratinocyte-derived chemokine; IBA-1, ionized calcium binding adaptor molecule 1; IFN-γ, interferon γ; IL, interleukin; i.p., intraperitoneal injection; LPS, lipopolysaccharide; MAPK 8, mitogen-activated protein kinase 8; MCP-1, monocyte chemoattractant protein-1; M-CSF, macrophage colony-stimulating factor; MIP, macrophage inflammatory protein; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; NGS, normal goat serum; NPS, new psychoactive substances; PBS, phosphate-buffered solution; RANTES, regulated on activation, normal T cells expressed and secreted; SCRA, synthetic cannabinoid receptor agonist; SD, Sprague–Dawley; THC, Δ^9-Tetrahydrocannabinol; TNF-α, tumor necrosis factor α; VEGF, vascular endothelial growth factor; VTA, ventral tegmental area.

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Addiction (EMCDDA) (EMCDDA, 2022). SCRAs available on the market are commonly sprayed on herbal material (or tobacco), or sold as e-liquid for use in electronic cigarettes. Recently, low-content cannabis and cannabis-based products have been found to be adulterated with SCRAs throughout Europe, representing a new and dangerous threat to health (EMCDDA, 2022). Compared to Δ^9-tetrahydrocannabinol (THC), the principal psychoactive constituent of cannabis, SCRAs are potent full agonists of brain cannabinoid receptors (CBRs), leading to a longer duration of action and more extreme adverse effects than THC (Cohen and Weinstein, 2018; Palamar et al., 2017).

Considered the prototypical compound of the so-called 'first generation' class of SCRAs, JWH-018 is a full agonist of cannabinoid CB1R and CB2R exhibiting higher potency and affinity than THC for CB1R and producing in vivo cannabimimetic effects. Many of the cannabimimetic effects of acute JWH-018 exposure measured in rodents include antinociception, hypothermia, catalepsy, and locomotor suppression (Brents et al., 2011; Vigolo et al., 2015; Wiley et al., 2012). Recently, we showed that repeated exposure to a low-moderate dose of JWH-018 (0.25 mg/kg, intraperitoneal injection, i.p.) in adult male rats recapitulates some of the typical effects of drug dependence such as alteration of dopamine (DA) transmission and its responsiveness to motivational taste stimuli, affective behaviors, and CB1R downregulation in mesocorticolimbic DA circuits (Pintori et al., 2021).

In recent decades, several pieces of evidence have demonstrated that glial cells (microglia and astrocytes) are markedly influenced by exposure to different classes of drugs of abuse, exhibiting significant changes in morphology, gene expression, and function (see Lacagnina et al., 2017). Moreover, drug-induced microglial activation elicits the release of proinflammatory cytokines, chemokines, and colony-stimulating factors (CSFs), which may result in neurotoxicity, neurodegeneration, and/or cognitive dysfunction (Lisboa et al., 2018; Liu et al., 2016). Exposure to THC or SCRAs induces alterations in glial cells and brain cytokine production (Bisogno and Di Marzo, 2010; Moretti et al., 2015; Walter and Stella, 2004), suggesting that the endocannabinoid system plays a crucial role in neuroinflammation. In this regard, CB1Rs and CB2Rs are present in both microglia and astrocytes (Navarrete and Araque, 2008; Stella, 2010), and their expression depends on the microglial activation profile. Further evidence supports the anti-inflammatory role of endogenous, natural, and synthetic cannabinoids, which can downregulate inflammatory and upregulate anti-inflammatory cytokines acting at CB2Rs (Klein et al., 2000; Massi et al., 2006; Ranieri et al., 2016).

Preclinical and clinical studies of plasma and brain samples demonstrate that repeated exposure to THC reduces expression of the anti-inflammatory cytokine interleukin (IL)-10 while increasing microglial reactivity and the levels of proinflammatory cytokines including IL-1β, IL-6, IL-8, tumor necrosis factor (TNF)α (Bayazit et al., 2017; Cutando et al., 2013; Zamberletti et al., 2015). By comparison, most published studies evaluating the effect of SCRAs on cytokine profiles have focused primarily on peripheral tissues. For example, Zawatsky and colleagues (Zawatsky et al., 2020) demonstrated in mice that oropharyngeal CP55,940 administration significantly increases CB1R and CB2R gene expression, together with an increased expression of inflammatory cytokines (TNF-α, IL-1β, and IL-6) and chemokines (chemokine C-C—ligand 2 and 3, chemokine C-X ligand 1 and 10) in the lung. Kevin and colleagues (Kevin et al., 2017) reported decreased IL-1α and IL-12 plasma levels in rats after adolescent treatment with AB-FUBINACA. Recently, our group demonstrated that adolescent voluntary consumption of JWH-018 leads to long-lasting behavioral changes, neurochemical dysregulation, and glial cell activation (i.e., astrocytopathy and microgliosis) (Margiani et al., 2023). Glial alterations in adult brains were coupled with increased chemokine regulated on activation normal T cells expressed and secreted (RANTES), decreased IL-2 and IL-13 in the cortex, and increased monocyte chemoattractant protein-1 (MCP-1) in the striatum (Margiani et al., 2023). In addition to behavioral and neurochemical alterations, we observed that repeated JWH-018 exposure (0.25 mg/kg, i.p.) in adult rats produces an increase in ionized calcium binding adaptor molecule 1 (IBA-1)-positive cells and glial fibrillary acidic protein (GFAP) immunoreactivity in the medial prefrontal cortex (mPFC), ventral striatum (i.e., nucleus accumbens (NAc) shell and core) and ventral tegmental area (VTA) 24 hours after treatment discontinuation (Pintori et al., 2021). Importantly, contemporary astrogliosis and microgliosis persisted up to seven days selectively in the NAc after JWH-018 discontinuation, suggesting SCRAs have a robust impact in this region.

In the present paper, we aimed to investigate the role of repeated JWH-018 exposure on cytokine, chemokine, and growth factor expression, which could be correlated with our previously reported behavioral and cellular effect (Pintori et al., 2021). To this end, adult male Sprague–Dawley (SD) rats were exposed to JWH-018 (0.25 mg/kg, i.p.) for fourteen consecutive days, and seven days after treatment discontinuation, we analyzed the full profile of cytokines using the Bio-Plex Multiplex-Immunoassay-System in the cortex, hippocampus, and striatum. Markers of neuroinflammation for microglia (IBA-1) and astrocytes (GFAP) were evaluated in the Caudate-Putamen (CPu), and in combination with previous work evaluating the NAc (Pintori et al., 2021) allowed for a comprehensive analysis of cytokine changes within the striatum.

2. Material and methods

2.1. Animals

Twenty-four adult male SD rats (275–300 g) were used. Animals were housed in groups of six in standard plastic cages with wood chip bedding at a temperature of 22 ± 2 °C and 60% humidity under a 12 h light/dark cycle (lights on from 7:00 a.m.). 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 are reported in compliance with the Guidelines for Care and Use of Mammals in Neuroscience and Behavioral Research according to Italian (D.Lgs 26/2014) and European Council Directive (2010/63/UE) and to the guidelines issued by the Committee for Animal Wellbeing (OPBA) at the University of Cagliari. We made all efforts to minimize pain and suffering and to reduce the number of animals used, according to the 3Rs principles.

2.2. Drugs

JWH-018 (1-pentyl-3-(1-naphthoyl) indole, Tocris, Bristol, UK) was solubilized in 0.5% EtOH, 0.5% Tween 80, and 99% saline. JWH-018 or vehicle was administered intraperitoneally in a volume of 3 ml/kg of body weight.

2.3. Experimental design timeline

Rats were injected once a day with either JWH-018 (0.25 mg/kg i.p.) or vehicle for fourteen consecutive days (Pintori et al., 2021). Seven days after the last JWH-018 injection, rats were sacrificed to collect brain areas for either cytokine or immunohistochemical assays. Throughout the course of the experimental protocol (i.e., treatment and 7-days abstinence period), rats were housed in standard conditions (see paragraph 2.1) and were not involve in any activity. The experimental group sizes ($n \geq 5$) were chosen based on our previous experimental protocols (Pintori et al., 2021) and are shown in the figure legends. Due to technical issues (e.g., tissue preparation), some animals/samples were excluded from statistical analysis, thus reducing the group size in a few cases. Data evaluation and analysis were performed by blinded experimenters.

2.4. Cytokine assays

Seven days after the last JWH-018 injection, seven rats per group were deeply anesthetized with isoflurane (5%) and sacrificed by decapitation. Their brains were rapidly removed, and cerebral cortices, striatum, and hippocampus were dissected on ice, snap-frozen in liquid nitrogen and stored at −80 °C.

2.4.1. Sample processing

Briefly, frozen tissues were thawed and disrupted in Bioplex cell lysis buffer (Bio-Rad, Hercules, CA, USA) containing factors 1 and 2 (protease and phosphatase inhibitors, respectively; Bio-Rad) and the protease inhibitor phenyl-methylsulfonyl fluoride (500 mM; Sigma–Aldrich). Samples were homogenized on ice with a potter (Quiminigen, Portugal), and the homogenate was frozen for 30 min at −80 °C. After thawing and sonication for 15 s, the samples were centrifuged at 4 °C and 4500 ×*g* (Thermo Scientific Sorvall Legend Micro 17R Refrigerated Centrifuge) for 10 min. The supernatant was collected, and the protein content of each sample was determined using the Bio-Rad Protein Assay (Bio-Rad), with bovine serum albumin (BSA) as a standard, according to the manufacturer's protocol. The protein concentrations were determined according to the Bradford method using the Bio-Rad DC protein assay kit (Bio-Rad). Lysates were stored at −80 °C until the day of the experiment.

2.4.2. Measurements

Cortex, striatum, and hippocampus cytokine concentrations were measured using a Luminex xMAP-based multiplex bead-based immunoassay, the Bio-Plex ProTM Rat Cytokine Group I Panel 23-Plex (Bio-Rad Laboratories, Inc., USA), which detects cytokines: Interleukins (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL- 6, IL-7, IL-10, IL-12(p70), IL-13, IL-17 IL-18, interferon (IFN)γ, tumor necrosis factor (TNF)α; chemokines: monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)1α, MIP-3α, regulated on activation normal T cells expressed and secreted (RANTES), keratinocyte derived chemokine (GRO/KC/CXCL1), and growth factors: granulocytes macrophage colony-stimulating factor (GM-CSF), granulocyte (G-CSF), macrophage (M-CSF) and vascular endothelial growth factor (VEGF). Assays were performed in 96-well plates; briefly, each assay plate layout consisted of eight standards in duplicate, two blank wells and up to 39 tissue homogenate samples run in duplicate at 500 μg proteins/well. Samples from the vehicle- and JWH-018-treated animals were analyzed on the same plate. All wash steps were performed on a Bio-Plex Pro wash station at room temperature. A Bio-Plex MagPix Multiplex System by Luminex was used to read the plate, and data were analyzed using Bio-Plex Manager 4.1 software with 5-parametric logistic regression (5PL) curve fitting to determine the standard curve (pg/ml) from eight standards in duplicate and extrapolate the sample concentrations from the standard curve. Only standards and samples with coefficients of variance under 5% were used. When readings fell below the detection limit (below the background value), they were excluded from analyses.

2.5. Immunohistochemistry assays

Seven days after the last JWH-018 injection, five rats per group were deeply anesthetized with isoflurane (5%) and then transcardially perfused with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate-buffered solution (PBS), pH 7.4. The brains were rapidly removed and postfixed in the same fixative overnight. After repeated washing in 0.1 M PBS, brains were cryoprotected in 30% sucrose in PBS for 48 h. Immunostaining was performed on free-floating coronal sections (40 μm), which were obtained using a cryostat at levels comprising the Caudate-Putamen (AP: +1.70 to −0.30) according to the Rat Brain Atlas coordinates (Paxinos and Watson, 2007).

2.5.1. Sample processing

GFAP and IBA-1 staining were carried out as previously described (Castelli et al., 2014; Pintori et al., 2021). Briefly, free-floating sections were incubated at 4 °C for 24 h with a primary mouse monoclonal anti-GFAP antibody (1:5000; Millipore Cat# MAB360, RRID: AB_11212597) or with a rabbit monoclonal anti-IBA-1 antibody (1:2000; FUJIFILM Wako Shibayagi Cat# 016–20,001, RRID: AB_839506) in PBS containing 0.2% Triton X-100, 0.1% bovine serum albumin (BSA), and 1% normal goat serum (NGS). Then, the sections were washed in PBS containing 0.2% Triton X-100 and incubated with secondary antibodies for GFAP and IBA-1, Alexa Fluor 594-labeled goat anti-mouse IgG (for GFAP, 1:500; cat no. A-11005, Molecular Probes, USA) and Alexa Fluor 488-labeled goat anti-rabbit IgG (for IBA-1, 1:500; cat no. A-11012 Molecular Probes, USA) for 1 h in the dark at room temperature.

2.5.2. Measurements

Images were taken on an Olympus IX 61 microscope at 20× magnification, and the Cell P Analysis software module was used either for semiquantitative fiber density analysis (GFAP) or cell number analysis (IBA-1). Analysis of GFAP and IBA-1 immunoreactivity (IR) was performed on the whole area (one tissue section out of every three successive sections), for a total of sixteen sections containing the CPu. GFAP-IR was expressed as average values of percentage (%) of the area occupied by fibers ± SEM, while the number of IBA-1 cells was expressed as the mean/mm^2 ± SEM.

2.6. Statistical analysis

All numerical data are given as the mean ± SEM. Data were tested for a normal distribution using Shapiro–Wilk's test. Data were collected from individual animals (independent determinations), and molecular changes produced by repeated JWH-018 exposure were analyzed separately for each brain area using an unpaired two-tailed Student's *t*-test. Unpaired t-test with Welch's correction was chosen when there was significant variance in homogeneity. Differences were considered significant at $p < 0.05$. All analyses were performed using the GraphPad software package (Prism, version 9; GraphPad, San Diego, California, USA).

3. Results

3.1. Effect of repeated JWH-018 exposure on cytokine expression seven days after JWH-018 discontinuation

To determine whether repeated JWH-018 exposure affects the expression of cytokines, chemokines, and growth factors, seven days after JWH-018 discontinuation, we performed a multiplex ELISA on selected brain areas (cortex, striatum, hippocampus) from vehicle- and JWH-018-treated rats. The high sensitivity of this approach allowed us to simultaneously measure the levels of 23 cytokines, chemokines, and growth factors from different brain areas. All these factors analyzed were measurable in the brain areas examined, except for IL-1β, which was undetectable within any of the selected brain areas. Table 1 shows the levels of all cytokines, chemokines, and growth factors in the striatum, cortex, and hippocampus of vehicle- and JWH-018-treated rats. Most of the cytokine concentrations range from 1 to 100 pg/ml. Cytokines and chemokines with the lowest concentrations in the striatum, cortex, and hippocampus included IL-1α, IL-4, IL-5, IL-7, IL-10, IL-13, IL-17, IFN- γ, MIP-1α, MIP-3α, GRO/KC, and RANTES. Conversely, cytokines with the highest concentrations (100–350 pg/ml) in these brain regions included IL-12(p70), IL-6, IL-2, IL-18, TNF-α, MCP-1, and the growth factor VEGF. GM-CSF, G-CSF, and M-CSF were present at low concentrations ranging from 4.95 to 24.35 pg/ml. Most cytokine and chemokine profiles were similar across the three brain areas. This is most clearly seen for the chemokines, where the profiles for each chemokine (except for MCP-1 in the striatum) in the three brain areas were nearly identical (Table 1). As shown in Fig. 1, repeated JWH-018 exposure altered cytokine expression in the striatum but not in the

Table 1
Neuroimmune signaling profile of the striatum, cortex, and hippocampus of rats after repeated JWH-018 exposure.

	Striatum		Cortex		Hippocampus	
	Veh	JWH-018	Veh	JWH-018	Veh	JWH-018
Cytokines (pg/ml)						
IL-1α	27.23 ± 1.16	30.37 ± 0.88	25.52 ± 1.52	23.33 ± 1.21	23.93 ± 0.60	20.81 ± 1.77
IL-1β	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
IL-2	214.51 ± 9.56	247.93 ± 8.16*	325.40 ± 25.01	346.9 ± 14.46	233.40 ± 22.93	271.30 ± 20.90
IL-4	16.86 ± 0.53	20.57 ± 0.78**	17.26 ± 0.98	15.87 ± 0.53	15.77 ± 0.92	13.78 ± 1.02
IL-5	88.07 ± 3.16	96.31 ± 2.39	74.01 ± 5.03	68.45 ± 1.64	73.55 ± 2.60	64.53 ± 5.36
IL-6	263.70 ± 11.98	288.70 ± 6.88	207.00 ± 7.76	204.6 ± 3.74	200.12 ± 10.12	190.12 ± 10.18
IL-7	33.58 ± 2.98	36.39 ± 1.51	25.00 ± 1.05	25.17 ± 0.19	22.87 ± 1.34	21.88 ± 0.89
IL-10	110.3 ± 4.45	112.70 ± 3.83	93.23 ± 2.22	93.47 ± 0.57	84.26 ± 4.85	79.98 ± 2.86
IL-12 (p70)	150.30 ± 8.51	183.23 ± 5.44**	117.90 ± 6.50	110.70 ± 2.20	133.00 ± 3.65	111.12 ± 9.70
IL-13	62.65 ± 2.44	71.02 ± 2.35*	50.13 ± 4.11	46.51 ± 0.87	50.06 ± 2.52	42.89 ± 4.56
IL-17	49.63 ± 2.82	57.58 ± 3.13	41.30 ± 3.79	37.17 ± 0.79	40.92 ± 3.74	35.84 ± 3.02
IL-18	120.00 ± 3.45	129.10 ± 3.41	112.80 ± 4.81	108.50 ± 1.91	111.10 ± 3.94	110.80 ± 2.25
IFN-γ	92.10 ± 3.59	109.40 ± 4.17**	89.26 ± 4.70	87.17 ± 1.61	84.57 ± 5.58	78.89 ± 5.33
TNF-α	132.10 ± 10.89	132.00 ± 6.43	116.30 ± 3.56	118.10 ± 4.03	95.05 ± 5.75	106.00 ± 4.72
Chemokines (pg/ml)						
MCP-1	215.60 ± 9.54	241.90 ± 14.32	154.10 ± 8.00	155.40 ± 4.29	148.10 ± 7.31	139.60 ± 4.55
MIP-1α	8.42 ± 0.21	10.06 ± 0.60*	8.58 ± 0.37	8.36 ± 0.24	7.31 ± 0.21	6.80 ± 0.30
MIP-3α	8.38 ± 0.52	10.28 ± 0.40*	9.18 ± 0.69	8.50 ± 0.34	7.63 ± 0.38	6.50 ± 0.60
RANTES	31.29 ± 1.23	33.95 ± 1.21	25.74 ± 1.33	24.58 ± 0.57	24.42 ± 1.01	22.03 ± 1.29
GRO/KC	25.16 ± 0.92	27.81 ± 0.97	22.73 ± 1.20	22.23 ± 0.64	27.68 ± 1.23	25.59 ± 1.84
Growth factors (pg/ml)						
GM-CSF	39.00 ± 3.10	43.68 ± 1.72	24.31 ± 1.18	23.88 ± 0.33	23.90 ± 1.41	22.25 ± 1.05
G-CSF	18.34 ± 1.21	19.01 ± 1.21	9.43 ± 0.68	9.52 ± 0.25	10.46 ± 0.41	9.57 ± 0.31
M-CSF	5.77 ± 0.35	7.30 ± 0.33**	4.95 ± 0.42	4.35 ± 0.11	4.84 ± 0.17	4.39 ± 0.38
VEGF	345.90 ± 18.80	408.60 ± 16.56*	291.10 ± 19.50	273.80 ± 2.95	273.50 ± 17.61	240.10 ± 21.07

Data, expressed as a pg/ml, are mean ± SEM of cytokines, chemokines, and growth factors levels in the striatum, cortex, and hippocampus of rats seven days after JWH-018 discontinuation, calculated from one experiment performed in duplicate. * $p < 0.05$, ** $p < 0.01$ Veh vs JWH-018. Unpaired two-tailed Student's t-test with or without Welch's correction Veh: $n = 5$–6 rats; JWH-018: $n = 6$–7 rats.

Abbreviations: N.D., not detectable: cytokines, chemokines, or growth factors under the limit of detection. IL, Interleukins; IFN, interferon; TNF, tumor necrosis factor; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cells expressed and secreted; GRO/KC/CXCL1, keratinocyte derived chemokine; GM-CSF, granulocytes macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; M-CSF macrophage colony-stimulating factor; VEGF, vascular endothelial growth factor.

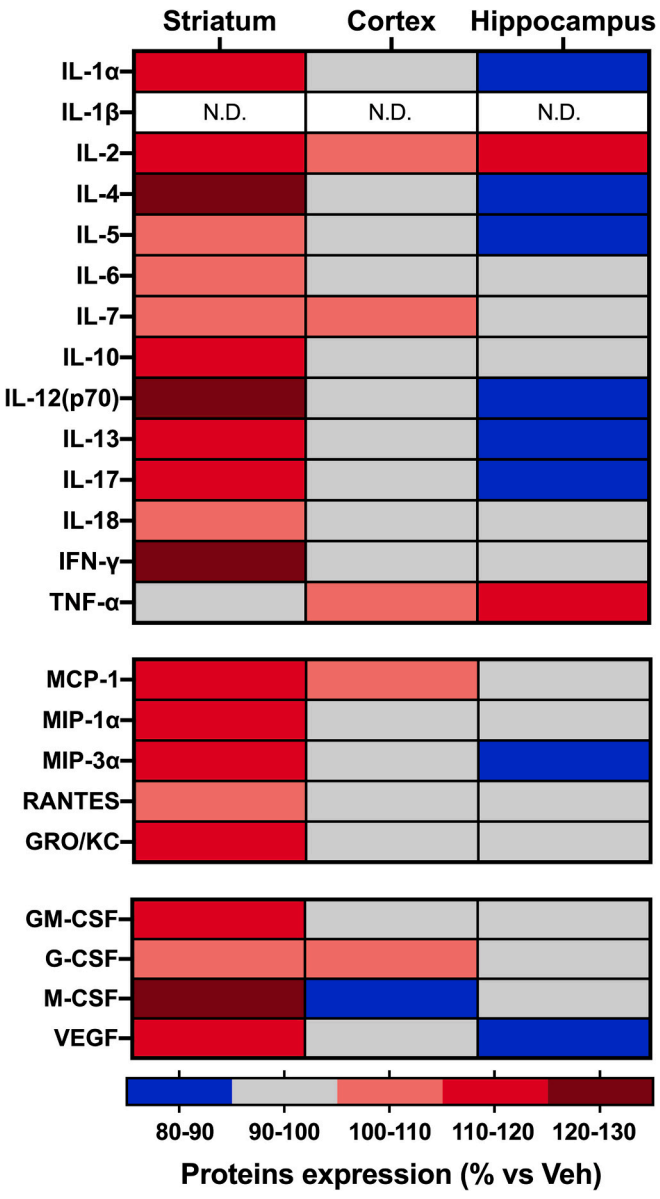

Fig. 1. Neuroimmune signaling profile in the striatum, cortex, and hippocampus of rats after repeated JWH-018 exposure.
Heatmap reports the expression of cytokines, chemokines, and growth factors in the striatum, cortex, and hippocampus of JWH-018-treated rats seven days after JWH-018 discontinuation. Data are expressed as percentages (%) of the Veh group. The degree of change is indicated by the depth of color shown on the right label: blue, 80–90%; gray, 90–100%; light red, 100–110%, red, 110–120%, dark red, 120–130%. Veh: $n = 5$–6 rats; JWH-018: $n = 6$–7 rats. Abbreviations: N.D., not detectable: cytokines, chemokines, or colony-stimulating factors under the limit of detection. IL, Interleukins; IFN, interferon; TNF, tumor necrosis factor; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cells expressed and secreted; GRO/KC/CXCL1, keratinocyte derived chemokine; GM-CSF, granulocyte macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; M-CSF macrophage colony-stimulating factor; VEGF, vascular endothelial growth factor. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cortex or hippocampus. In particular, Fig. 2 and Fig. 3 show significantly increased levels of the cytokines IL-2 ($t_{(10)} = 2.654$; $p = 0.0242$; + 16%), IL-4 ($t_{(11)} = 3.812$; $p = 0.0029$; + 22%), IL-12p70 ($t_{(11)} = 3.359$; $p = 0.0064$; + 22%), IL-13 ($t_{(11)} = 2.461$; $p = 0.0316$; + 13%), IFN-γ ($t_{(11)} =$

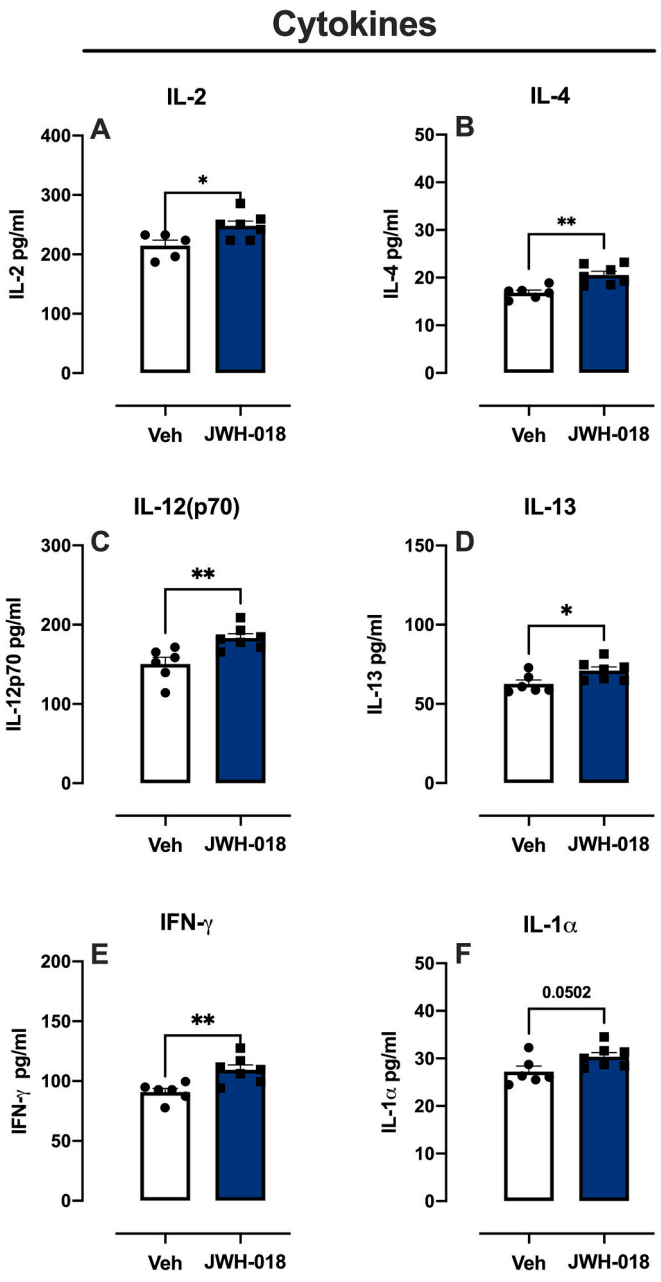

Fig. 2. Effect of repeated JWH-018 exposure on cytokine expression in the striatum seven days after JWH-018 discontinuation. Data are presented as the mean ± SEM of IL-2 (A), IL-4 (B), IL-12p70 (C), IL-13 (D), and IL-1α (F), expressed as pg/ml. * $p < 0.05$, ** $p < 0.01$ Veh vs JWH-018. Unpaired two-tailed Student's *t*-test. Veh: n = 5–6 rats; JWH-018: $n = 7$ rats.

3.464; $p = 0.0053$; + 20%) (Fig. 2A-E), the chemokines MIP-1α ($t_{(6.21)} = 2.581$; $p = 0.0404$; + 20%), MIP-3α ($t_{(11)} = 2.833$; $p = 0.0163$; + 17%) (Fig. 4A-B), and the growth factors VEGF ($t_{(10)} = 2.502$; $p = 0.0313$; + 18%) and M-CSF ($t_{(11)} = 3.770$; $p = 0.0031$; + 27%) (Fig. 3C-D) in the striatum of JWH-018 exposed rats. Notably, a tendency to increase IL-1α expression was observed in the striatum of JWH-018-treated rats ($t_{(11)} = 2.198$; $p = 0.0502$; + 12%) (Fig. 2F). No significant differences in the levels of either cytokines, chemokines or growth factors between groups were observed in either the cortex or hippocampus (Table 1, Fig. 1). These findings suggest that JWH-018-induced cytokine perturbation is brain region specific.

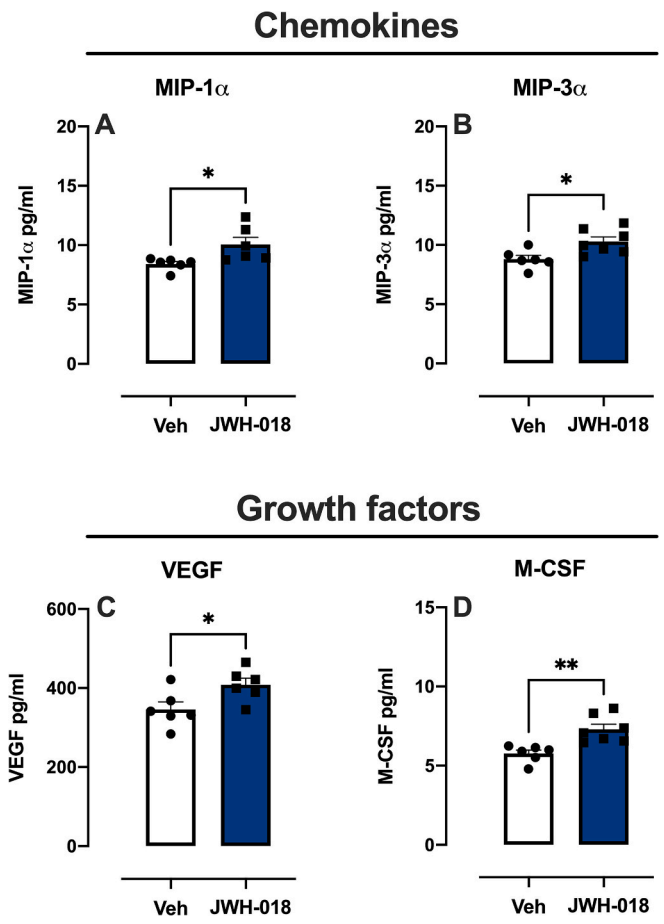

Fig. 3. Effect of repeated JWH-018 exposure on chemokine and growth factors expression in the striatum seven days after JWH-018 discontinuation. Data are presented as the mean ± SEM of MIP-1α (A), MIP-3α (B), VEGF (C), and M-CSF (D), expressed as pg/ml. * $p < 0.05$, ** $p < 0.01$ Veh vs JWH-018. Unpaired two-tailed Student's t-test with or without Welch's correction. Veh n = 6 rats; JWH-018 n = 6–7 rats.

3.2. Effect of repeated JWH-018 exposure on GFAP and IBA-1 IR seven days after JWH-018 discontinuation

We recently demonstrated that repeated JWH-018 exposure induces microgliosis and astrogliosis in several DAergic brain areas, such as the mPFC, ventral striatum (NAc shell and core), and VTA. While astrogliosis persisted in all these brain areas, microgliosis was observed only in the NAc up to seven days after JWH-018 discontinuation (Pintori et al., 2021). To determine whether the altered striatal profile of cytokines induced by JWH-018 was associated with changes in glial cells (astrocytes and microglia) in both the ventral and dorsal striatum, we evaluated the expression of GFAP and IBA-1 IR in the dorsal striatum (CPu) after JWH-018 discontinuation.

As shown in Fig. 4, both GFAP and IBA-1 IR levels, markers of astrogliosis and activated microglia, were increased in the CPu seven days after repeated JWH-018 exposure. Specifically, Student's *t*-test showed increased expression of GFAP IR and IBA-1 IR in JWH-018-treated rats compared to vehicle-treated rats (GFAP: $t_{(8)} = 2.596$, $p < 0.0244$; + 26%, Fig. 4A; IBA-1: $t_{(8)} = 2.767$, $p < 0.0318$; + 38%, Fig. 4B). Consistent with our previous findings (Pintori et al., 2021), these results demonstrated that repeated JWH-018 exposure induces protracted astrogliosis and microgliosis in the whole striatum.

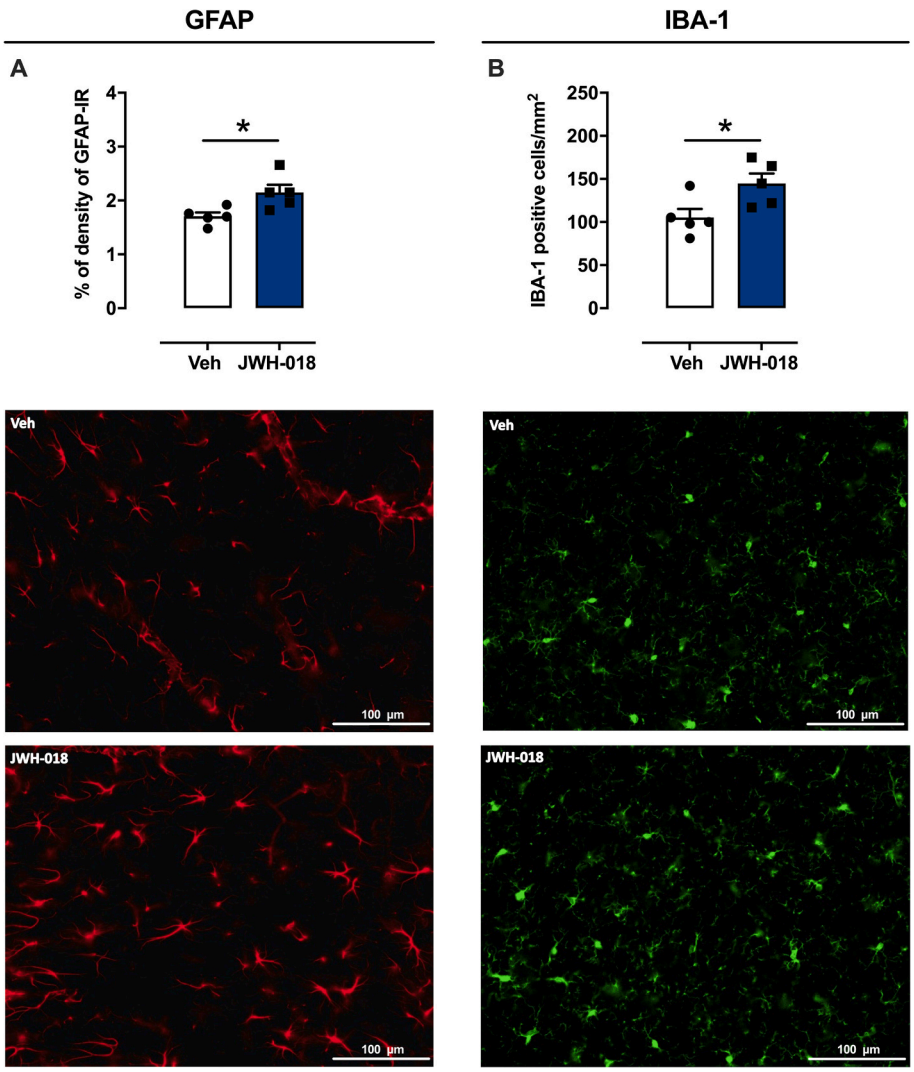

Fig. 4. Effect of repeated JWH-018 exposure on GFAP and IBA-1 immunoreactivity (IR) in the caudate putamen (CPu) seven days after JWH-018 discontinuation. Data are presented as the mean ± SEM of density reading expressed as a percentage of the area covered by GFAP-IR (A) and of the number of IBA-1-positive cells expressed per mm^2 (B). Representative images of GFAP (left) and IBA-1 (right) expression in Veh- and JWH-018-treated rats. * $p < 0.05$ Veh vs JWH-018. Unpaired two-tailed Student's t-test. n = 5 rats per group.

4. Discussion

The present study demonstrates that repeated JWH-018 exposure causes striatal-specific perturbation of cytokines in the brain associated with microgliosis and astrogliosis seven days after drug discontinuation. Specifically, JWH-018 induces elevation in proinflammatory cytokines (IL-2, IL-4, IL-12p70, IFN-γ, and IL-13), chemokines (MIP-1α and MIP-3α) and growth factors (M-CSF and VEGF). Moreover, the increased IBA-1 and GFAP expression observed in the CPu extends our previous findings in the NAc (Pintori et al., 2021) to show that JWH-018 induces microgliosis and astrogliosis of throughout the striatum.

To the best of our knowledge, few studies have evaluated the impact of SCRAs on cytokine expression. However, most of them have assessed only *in vitro* the cytotoxicity and neuroinflammatory effects induced by these compounds. For instance, CP-47497-C8 increases IL-12(p40), IL-6, and TNF-α levels in human lymphocytes stimulated with lipopolysaccharide (LPS) (Bileck et al., 2016). Koller and colleagues evaluated the immunomodulatory activities of four naphthoylindole-based SCRAs (JWH-018, JWH-073, JWH-122, and JWH-210) and one benzoylindole-based SCRA (AM-694) in human cell lines and primary cells (Koller et al., 2013). Additionally, it has been reported that JWH-210 and JWH-122 decrease TNF-α and IL-12/23p40 release in LPS-activated peripheral blood mononuclear cells. Otzas and colleagues (Oztas et al., 2019) investigated *in vitro* the inflammatory activity of AKB48, a third-generation SCRA. The authors observed an upregulation of mitogen-activated protein kinase 8 (MAPK 8) gene expression only with 25 μM AKB48, while the levels of TNF-α and IL-6 were upregulated in a dose-dependent manner. Finally, the only case-control study available in SCRA abusers showed mononuclear leukocyte DNA damage, increased plasma total oxidant status, and increased levels of IL-1β, IL-6, and TNF-α (Guler et al., 2020). Of note, only our group investigated in animal models the neuroinflammatory effects of active (self-administration; see Margiani et al., 2023) and passive (experimenter given, i.p. injection; see Pintori et al., 2021) JWH-018 exposure. In particular, we demonstrated that self-administration of JWH-018 during adolescence induces long-term microglia and astrocyte alterations (i.e., increased IBA-1 and decreased GFAP expression) specifically in the CPu (Margiani et al., 2023). This glial perturbation was accompanied by persistant dysregulation of cytokines and chemokines in the cortex (i.e., decreased IL-2, IL-13, and RANTES levels) and in the striatum (i.e., increased MCP-1 levels) of adult mice that self-administered JWH-018 during adolescence. Moreover, we recently reported that repeated passive JWH-018 exposure in adult male rats – using the same experimental protocol of our current study – increased GFAP and IBA-1 levels in several DA brain

areas (mPFC, NAc shell and core, VTA) 24 hours h after drug discontinuation (Pintori et al., 2021). Astrogliosis and microgliosis were only observed in the ventral striatum after seven days of JWH-018 discontinuation in both passive treated animals (Pintori et al., 2021) and in adult mice after adolescent JWH-018 self-administration (Margiani et al., 2023).

In the present study, using the passive JWH-018 exposure, we demonstrated that this kind of exposure also influences neuroimmune signaling, upregulating pro-inflammatory (IL-2, IL-12, and IFN-γ) and anti-inflammatory cytokine (IL-4 and IL-13), chemokine (MIP-1α, MIP-3α), and growth factor (M-CSF, VEGF) expression in the striatum but not in the cortex and hippocampus seven days after drug discontinuation. We observed increased GFAP and IBA-1 levels in the dorsal striatum (current study) as well as in the NAc (Pintori et al., 2021), demonstrating that JWH-018-induced neuroinflammation involves the whole striatum. The cytokines/chemokines released by activated macrophages are related to each other and modulate the immune response (Griffith et al., 2014; Liu et al., 2021). On the other hand, prolonged exposure to drugs of abuse induces neuroinflammation that activates microglia and astrocytes, which in turn increase cytokine and chemokine release (Cui et al., 2014). Therefore, our findings are consistent with cytokine/chemokine alterations that result from exposure to other drugs of abuse (Ahearn et al., 2021; Araos et al., 2015; Cui et al., 2014), confirming the important relationship between drug abuse and neuroinflammation.

We found increased expression of the pro-inflammatory cytokines IL-2, IL-12, and IFN-γ in the striatum but not in the cortex or hippocampus after JWH-018 discontinuation. In agreement with the present data, increased levels of pro-inflammatory IL-2 were found in hypothalamic samples of methamphetamine-treated mice seven days after the last administration (Loftis et al., 2013) and in the brains of male rats that self-administer the cathinones α-PVP and 4MMC (Marusich et al., 2022). In contrast, other studies have showed a decrease in IL-2 i) in the cortex of adult mice after adolescent self-administration of JWH-018 (Margiani et al., 2023), ii) in the plasma samples collected from adult rats treated in adolescence with AB-Fubinaca (Kevin et al., 2017), and iii) in blood samples collected from 3,4-MDMA and cannabis users (Pacifici et al., 2007). In the context of drug addiction, limited information is available on the role of IFN-γ and IL-12. Consistent with our findings, IFN-γ mRNA and protein levels were increased in the striatum of mice treated with paramethoxymethamphetamine 1 hour h, 2 hours h, one day, and seven days after the last treatment (Shin et al., 2016). Moreover, increased levels of IFN-γ, IL-6, and MCP-1 have been observed in the plasma from patients with a history of chronic alcohol overconsumption (Bjørkhaug et al., 2020). Several studies have demonstrated that these pro-inflammatory cytokines can modulate DA transmission as well as the underlying cognitive processes. While IL-2 increases DA release *in vitro* (Alonso et al., 1993; Lapchak, 1992), *in vivo* findings demonstrate that systemic IL-2 administration reduces DA levels in the NAc (Anisman et al., 1996). Work by Petitto and colleagues (Petitto et al., 1997) suggests that these discrepancies might be explained by differences in the local concentration of IL-2 in each context. Similarly, *in vitro,* IFN-γ can affect DA transmission, decreasing the uptake of the DA precursor (Vumma et al., 2017). Importantly, IFN-γ production is promoted by IL-12, which plays a critical role in bridging the innate and adaptive immune responses (Hamza et al., 2010).

In addition to the upregulation of pro-inflammatory molecules, we found increased levels of the anti-inflammatory cytokines IL-4 and IL-13 in the striatum of rats repeatedly exposed to JWH-018. The enhancement of these two cytokines can be considered a compensatory mechanism occurring in response to an excessive/harmful inflammatory response mediated by IL-2, IL-12, and IFN-γ. However, although both IL-4 and IL-13 are typically considered anti-inflammatory cytokines as they downregulate the synthesis of T-helper type 1 pro-inflammatory cytokine, they can also display neurotoxic effects by potentiating the effects of oxidative stress on neurons during neuroinflammation (Mori et al., 2016). For instance, Hong and colleagues (Hong et al., 2022) recently demonstrated the in vivo neurotoxic effects of IL-13. Here, striatal LPS injection increased IL-13 expression on microglia, which subsequently contributed to neurodegeneration through disruption of blood-brain barrier integrity and astrocyte damage in the striatum. Thus, we can hypothesize that prolonged/excessive glial activation might induce a dynamic balance between pro- and anti-inflammatory cytokines to recover from a chronic inflammatory state induced by JWH-018 treatment, which in turn drives neuronal homeostasis perturbations in the striatum.

Consistent with an enhancement of cytokine production, we observed an enhancement of the chemokines MIP-1α and MIP-3α and the growth factors M-CSF and VEGF levels in the striatum but not in the cortex and hippocampus. In addition to attracting immune cells to the site of inflammation, chemokines can also modulate neurotransmission between neurons and glial cells (Rostène et al., 2007; Watson et al., 2020). VEGF, which is involved in angiogenesis and vascular permeability, also plays an important role in nervous system activity and facilitates the development of microvasculature that supplies nutrients and oxygen in the brain (Lange et al., 2016). Thus, chemokines and VEGF perturbations have been associated with several neuropsychiatric diseases, including drug addiction (Ahearn et al., 2021; Stuart and Baune, 2014). For instance, increased VEGF serum levels were observed in humans during alcohol withdrawal (Heberlein et al., 2010), in alcohol use disorders (Requena-Ocaña et al., 2022), and in rats during two weeks of the onset of ethanol consumption (Louboutin et al., 2012). In agreement with the "neuroinflammatory hypothesis", we found an enhancement of M-CSF expression in the same brain area, which represents one of the principal candidates responsible for maintaining microglial activation (Imai and Kohsaka, 2002). Accordingly, we observed protracted and contemporary microglia and astrocyte activation (i.e., increased IBA-1 and GFAP expression) only in the ventral (Pintori et al., 2021) and dorsal (present findings) striatum, confirming that the magnitude of JWH-018-induced neuroinflammation is brain region specific. Due to the bidirectional communication between glial cells and DA neuron activity (Kettenmann et al., 2011; Petrelli et al., 2020), this striatal glial activation suggests an important contribution of neuroinflammatory processes in previously reported JWH-018-induced dopaminergic dysregulations (Pintori et al., 2021).

5. Limitations

This study was limited to evaluating profiles of a select subset of neuroimmune mediators (23 cytokines) from only three mesocorticolimbic brain regions. Additionally, molecular measurements were only performed at a single time point. Future studies will expand these findings to provide a better understanding of the impact of JWH-018 exposure and withdrawal on neuroinflammation by incorporating additional cytokines, brain sites, and timepoints during exposure and abstinence. Although our findings are in line with those reporting NPS-induced neuroinflammatory effects, comparisons of our data with the NPS and SCRAs literature should be interpreted with caution due to the different experimental conditions (i.e., *in vitro* vs. *in vivo*, rats vs. mice, passive vs. active administration, drug dosing, the time point of evaluations). Finally, neuroimmune signaling is strongly influenced by sex and further studies including female rats will likely uncover additional important data supporting the important role of neuroinflammation in drug addiction.

6. Conclusions

Our findings demonstrate that repeated JWH-018 exposure induces a protracted perturbation of neuroimmune signaling specifically in the striatum, which plays an important role in reward and reward-related processes. This neuroinflammatory fingerprint supports the hypothesis of an important involvement of neuroimmune signaling perturbation in the behavioral and neurochemical effects observed after repeated JWH-

018 exposure. The prolonged increase in pro- and anti-inflammatory cytokine, chemokine, and growth factor expression – along with concomitant microglia and astrocyte activation in the striatum – may contribute to DA and behavioral dysregulation. As a prototypical SCRA, the behavioral and neuroimmune responses observed with JWH-018 may be indicative of the effects produced by other synthetic cannabinoids.

Ethics approval

All animal care and experimental procedures are reported in compliance with the Guidelines for Care and Use of Mammals in Neuroscience and Behavioral Research according to Italian (D.Lgs 26/2014) and European Council Directive (2010/63/UE) and to the guidelines issued by the Committee for Animal Wellbeing (OPBA) at the University of Cagliari. We made all efforts to minimize pain and suffering and to reduce the number of animals used, according to the 3Rs principles.

Consent for publication

Not applicable.

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CRediT authorship contribution statement

Nicholas Pintori: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Rafaela Mostallino:** Writing – original draft, Investigation, Formal analysis, Data curation. **Enrica Spano:** Investigation. **Valeria Orrù:** Investigation. **Maria Grazia Piras:** Investigation. **Maria Paola Castelli:** Writing – original draft, Supervision, Resources, Formal analysis, Conceptualization. **Maria Antonietta De Luca:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

The data sets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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