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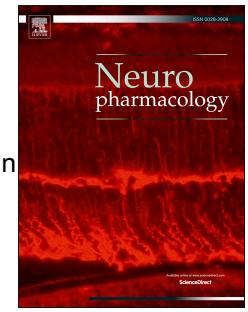

Stimulation OF *IN VIVO* dopamine transmission and intravenous self-administration in rats and mice by JWH-018, a Spice cannabinoid

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STIMULATION OF *IN VIVO* DOPAMINE TRANSMISSION AND INTRAVENOUS SELF-ADMINISTRATION IN RATS AND MICE BY JWH-018, A SPICE CANNABINOID

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Abstract

The synthetic cannabinoid 1-pentyl-3-(1-naphthoyl)-indole (JWH-018) has been detected in about 140 samples of a smokable herbal mixture termed "Spice". JWH-018 is a CB1 and CB2 agonist with a higher affinity than Δ^9-THC. In order to investigate the neurobiological substrates of JWH-018 actions, we studied by microdialysis in freely moving rats the effect of JWH-018 on extracellular dopamine (DA) levels in the nucleus accumbens (NAc) shell and core and in the medial prefrontal cortex (mPFC). JWH-018, at the dose of 0.25 mg/kg i.p., increased DA release in the NAc shell but not in the NAc core and mPFC. Lower (0.125 mg/kg) and higher doses (0.50 mg/kg) were ineffective. These effects were blocked by CB1 receptor antagonists (SR-141716A and AM 251) and were absent in mice lacking the CB1 receptor. *Ex vivo* whole cell patch clamp recordings from rat ventral tegmental area (VTA) DA neurons showed that JWH-018 decreases $GABA_A$-mediated post-synaptic currents in a dose-dependent fashion suggesting that the stimulation of DA release observed in vivo might result from disinhibition of DA neurons. In addition, on the "tetrad" paradigm for screening cannabinoid-like effects (i.e., hypothermia, analgesia, catalepsy, hypomotility), JWH-018, at doses of 1 and 3 mg/kg, i.p., produced CB1 receptor-dependent behavioural effects in rats. Finally, under appropriate experimental conditions, rats (20 μg/kg/inf iv, FR3; nose-poking) and mice (30 μg/kg/inf iv, FR1; lever-pressing) self-administer intravenously JWH-018. In conclusion, JWH-018 shares with the active ingredient of Marijuana, Δ^9-THC, CB1-dependent reinforcing and DA stimulant actions.

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Abbreviations

AM 251 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-(1-piperidyl)pyrazole-3-carboxamide

DA dopamine

GABA gamma-aminobutyric acid

IPSC inhibitory postsynaptic currents

i.p. intraperitoneal

i.v. intravenous

mPFC medial Prefrontal Cortex

MJ Marijuana

NAc Nucleus Accumbens

JWH-018 1-pentyl-3-(1-naphthoyl)indole

SA self-administration

SR141716A Rimonabant, 5-(4-Clorofenil)-1-(2,4-dicloro-fenil) - 4-metil-N-(piperidin-1-il)- 1H-pirazole-3-carbossammide

THC delta-9-tetrahydrocannabinol

VTA Ventral Tegmental Area

WIN WIN-55,212-2; (R)-(+)-[2,3-Dihydro-5-methyl-3-(4 morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone

Abstract

The synthetic cannabinoid 1-pentyl-3-(1-naphthoyl)-indole (JWH-018) has been detected in about 140 samples of a smokable herbal mixture termed "Spice". JWH-018 is a CB1 and CB2 agonist with a higher affinity than Δ^9-THC. In order to investigate the neurobiological substrates of JWH-018 actions, we studied by microdialysis in freely moving rats the effect of JWH-018 on extracellular dopamine (DA) levels in the nucleus accumbens (NAc) shell and core and in the medial prefrontal cortex (mPFC). JWH-018, at the dose of 0.25 mg/kg i.p., increased DA release in the NAc shell but not in the NAc core and mPFC. Lower (0.125 mg/kg) and higher doses (0.50 mg/kg) were ineffective. These effects were blocked by CB1 receptor antagonists (Rimonabant and AM 251) and were absent in mice lacking the CB1 receptor. *Ex vivo* whole cell patch clamp recordings from rat ventral tegmental area (VTA) DA neurons showed that JWH-018 decreases $GABA_A$-mediated post-synaptic currents in a dose-dependent fashion suggesting that the stimulation of DA release observed in vivo might result from disinhibition of DA neurons. In addition, on the "tetrad" paradigm for screening cannabinoid-like effects (i.e., hypothermia, analgesia, catalepsy, hypomotility), JWH-018, at doses of 1 and 3 mg/kg, i.p., produced CB1 receptor-dependent behavioural effects in rats. Finally, under appropriate experimental conditions, rats (20 μg/kg/inf iv, FR3; nose-poking) and mice (30 μg/kg/inf iv, FR1; lever-pressing) self-administer intravenously JWH-018. In conclusion, JWH-018 shares with the active ingredient of Marijuana, Δ^9-THC, CB1-dependent reinforcing and DA stimulant actions.

Keywords: JWH-018, CB1 receptor, dopamine, nucleus accumbens, microdialysis, electrophysiology

Running title: JWH-018 rewarding properties

1.0 INTRODUCTION

In the past decade a large number of "herbal mixtures" has been marketed as a legal alternative to Marijuana (MJ) (SAMHSA, 2012). These products are broadly known as "Spice", and have been sold under many different names (Spice Gold, nJoy, Blaze etc.) in "smart shops" and through the Internet since 2006. More than 140 different "Spice" products have been marketed, and despite efforts to make their sale and use illegal they continue to be easily obtained without age restriction. These products are usually made up of shredded plant material that does not contain psychoactive constituents but laced with a variety of synthetic drugs that activate the same receptors of Δ^9-THC, the psychoactive principle of MJ (i.e. cannabinoid CB1 and CB2 receptors).

"Spice" users report experiences similar to those produced by MJ, though their high psychoactive potency can result in greater withdrawal symptoms and more adverse effects than MJ (e.g. headache, vomiting, paranoia, tachycardia, hypertension, seizures, panic attacks, agitation, psychosis, hallucination extreme anxiety/panic, paranoia, hallucinations, etc.) (Every-Palmer, 2011; Seely et al., 2012; Papanti et al., 2013; Fantegrossi et al., 2014). This suggests a greater prevalence of dependence on "Spice" *vs* MJ and a much greater risk to health and social wellbeing than MJ. Accordingly, the European Monitoring Centre for Drugs and Drug Addiction has monitored Spice consumption and abuse (EMCDDA, 2009).

JWH-018 (1-pentyl-3-(1-naphthoyl) indole), a potent CB1 and CB2 synthetic agonist, has been frequently identified as a component of many different "Spice" drugs. When compared to Δ^9-THC, JWH-018 exhibits a full-agonist profile with an approximate fourfold affinity for the CB1 receptor (*Ki ~ 9 nM*) and tenfold affinity for the CB2 receptor (*Ki ~ 3 nM*) (Huffman et al., 2005). Most likely, the higher affinity to

CB receptors contributes to the greater incidence of dependence and withdrawal associated with "Spice" *vs* MJ use. Furthermore, *in vitro* studies carried out on both hepatic microsomes (Wintermeyer et al., 2010) and urine samples (Sobolesky et al., 2010) showed the presence of pharmacologically active metabolites of JWH-018, which might account for long term *in vivo* effects produced by the substance and homologues, in particularly after their repeated use (Brents et al., 2011; Seely et al., 2012). Drug-discrimination studies in rats have showed that JWH-018 and Δ^9-THC can be fully substituted, mutually (Wiley et al., 2014a). In mice, JWH-018 displays cannabimimetic activity as estimated by the induction of a characteristic tetrad profile of dose-dependent effects (Wiley et al., 2012; Vigolo et al., 2015). Such studies, however, have not been performed yet in rats.

It is well established that the rewarding properties of natural and drug stimuli are tightly coupled to brain dopamine (DA) neurotransmission (Schultz, 2002; Di Chiara et al., 2004; Wise, 2008). Drugs of abuse have been shown to increase DA transmission in the ventral striatum/nucleus accumbens (NAc) (Di Chiara, 1990; Volkow et al., 2003; Fillenz, 2005) and it has been hypothesized that this property, depending on the specific drug class, mediates certain specific properties of drugs of abuse and therefore are instrumental to their addictive properties (Di Chiara and Imperato, 1988; Koob and Bloom, 1988; Wise and Rompre, 1989; Volkow et al., 2012). Like other drugs of abuse, the activation of DA transmission by natural (Δ^9-THC) and synthetic (e.g. WIN 55.212-2) cannabinoids is specifically associated to the shell subdivision of the NAc (Tanda et al., 1997; Lecca et al., 2006; De Luca et al., 2012). In humans, Δ^9-THC induces DA release in the ventral striatum (Bossong et al., 2009) whereas Δ^9-THC and WIN 55.212-2 are self-administered by monkeys and rats, respectively (Justinova et al., 2003; Fattore et al. 2001; Lecca et al., 2006). These

behavioural effects result from stimulation of CB1 receptors and are thought to be mediated by pre-synaptic mechanisms on the ventral tegmental area (VTA); opioid-dependent disinhibition of GABAergic terminals by cannabinoids leads to increases of DA extracellular levels in the NAc shell (Tanda et al., 2007).

In spite of the popularity of "Spice" and JWH-018, only few data on the neurobiological mechanisms of their rewarding effects and abuse potential are available. To fill this gap, we investigated the effect of JWH-018 on DA transmission in the NAc shell and core and medial prefrontal cortex (mPFC) by *in vivo* microdialysis and *ex vivo* electrophysiological recordings. We also investigated the behavioural properties of JWH-018 and its ability to support intravenous self-administration.

2.0 MATERIALS AND METHODS

Detailed methods are provided in Supplement 1.

2.1 Animals and Surgeries

Male Sprague-Daweley rats, C57BL/6 mice and CB1 KO homozygous mutant mice ($CB1^{-/-}$) and wild type ($CB1^{+/+}$) littermates were employed for the studies. For the IV self-administration studies, animals were prepared with intravenous catheters in the right jugular vein. For the intracranial surgery, the animals underwent stereotaxic surgery in which microdialysis probes were implanted and aimed at the NAc shell and core and mPFC.

2.2 Drugs

JWH-018 and WIN-55212 were purchased from Tocris (Bristol, UK). Rimonabant hydrochloride (SR141716A) and AM 251 were purchased from RD-Sigma (Italy). Drugs were dissolved and administered as described in the supplementary materials.

2.3 Microdialysis

Dialysate samples (10 µl) were injected into an HPLC equipped with a reverse phase column (C8 3.5 um, Waters, Mildford, MA, USA) and DA has been quantified by a coulometric detector (ESA, Coulochem II, Bedford, MA).

2.4 Electrophysiology

Neurons were visualized using an upright microscope with infrared illumination (Axioskop FS 2 plus, Zeiss), and whole-cell patch-clamp recordings were performed by using an Axopatch 200B amplifier (Molecular Devices, CA).

2.5 Screening for cannabinoid-like effects *in vivo*

Tests for body temperature, nociception, and motor activity were performed after injection of vehicle, JWH-018 and AM 251. Tests were performed at different times post-injection depending on the effect under investigation, as previously described (Vigolo et al., 2015).

2.6 Statistical Analysis

All the numerical data are given as mean ± SEM. Data were analyzed by utilizing repeated measures ANOVA or T-test. Results from treatments showing significant overall changes were subjected to *post hoc* Tukey tests (*in vivo* microdialysis), to Bonferroni's or Dunnett's tests (electrophysiological studies and cannabinoid-like screening effects), with significance for $p < 0.05$. As to the self-administration studies, nose-pokes/lever-presses emitted during each session through acquisition and extinction phases were analyzed by two-way ANOVA with nose-pokes/lever-presses (i.e., active *vs.* inactive) and days as within factors. Reacquisition was analyzed by two-way ANOVA, with nose-pokes/lever-presses (i.e., active *vs.* inactive) and days in respect to the corresponding final JWH-018 session as factors. Results from treatments showing significant overall changes were subjected to *post hoc* LSD.

3.0 RESULTS

3.1 *IN VIVO* MICRODIALYSIS STUDIES

Rat basal values of DA, expressed as fmoles/10 µl sample (mean ± SEM), were: NAc shell 52 ± 5 (N = 55), NAc core 55 ± 4 (N = 26), mPFC 16± 2 (N = 29). Mouse basal values of DA, expressed as fmoles/20 µl sample (mean ± SEM), were: $CB1^{+/+}$ NAc shell 53±14 (N= 4), $CB1^{-/-}$ NAc shell 33±11 (N= 5).

3.1.1 Effect of JWH-018 administration on DA transmission in the NAc shell and core, and in the mPFC

In this first experiment, we studied the effect of three doses of JWH-018 (0.125, 0.25 and 0.5 mg/kg i.p.) on extracellular DA levels in NAc shell and core, and mPFC. As shown in Fig. 1, the dose-response curve of the effect of JWH-018 on dialysate DA is bell-shaped with the dose of 0.25 mg/kg i.p. increasing DA levels preferentially in the NAc shell as compared to the NAc core and mPFC. No significant effects were observed in the NAc core and mPFC. Three-way ANOVA showed a main effect of dose [$F_{(3,75)}$=4.46; $p < 0.01$], brain area [$F_{(2,75)}$=7.72; $p <0.001$] and time [$F_{(12,900)}$=4.24; $p < 0.001$], and a significant dose x brain area interaction [$F_{(6,75)}$=6.46; $p < 0.0001$]. Tukey's *post hoc* tests showed a larger increase of dialysate DA in the NAc shell after 0.25 mg/kg i.p. of JWH-018 as compared to basal, to vehicle, to the 0.125 and 0.5 mg/kg i.p. doses and to the same dose (0.25 mg/kg) in the NAc core, and mPFC.

3.1.2 Role of CB1 receptors on the NAc shell DA stimulation induced by JWH-018

In this set of experiments, we studied the effect of CB1 receptor blockade by inverse agonists/antagonists Rimonabant (SR141716A) and AM 251 on the NAc shell DA response to JWH-018 (0.25 mg/kg i.p.) in rats (Fig. 2A). The effect of CB1 genetic deletion on changes in NAc shell DA levels after JWH-018 administration was also

evaluated in CB1 KO mice (Fig. 2B). In Rimonabant pre-treated animals, two-way ANOVA showed a main effect of treatment [$F_{(1,13)}$=17.22; p< 0.005], of time [$F_{(12,156)}$=3.13; p < 0.0001], and treatment x time significant interaction [$F_{(12,156)}$=2.5; p < 0.001]. Tukey's *post hoc* tests revealed that pre-treatment with Rimonabant reduced dialysate DA in the NAc shell as compared to rats pre-treated with vehicle. Similarly, in AM 251 pre-treated animals, two-way ANOVA showed a main effect of treatment [$F_{(1,6)}$=28.32; p < 0.005], of time [$F_{(12,72)}$=4.0; p < 0.0001], and treatment x time significant interaction [$F_{(12,72)}$=2.75; p< 0.005]. Tukey's *post hoc* tests revealed that AM 251 pretreatment reduced dialysate DA in the NAc shell as compared to rats pretreated with vehicle (Fig. 2A). In addition, we studied the effect of JWH-018 administration (0.3 mg/kg i.p.) on extracellular DA release in NAc shell of homozygous mutant mice ($CB1^{-/-}$) and wild type ($CB1^{+/+}$) littermates. Two-way ANOVA showed a main effect of treatment [$F_{(1,7)}$=8.25; p < 0.05], of time [$F_{(9,63)}$=4.64; p < 0.0001], and treatment x time significant interaction [$F_{(9,63)}$=5.23; p < 0.0001]. Tukey's *post hoc* tests revealed that JWH-018 treatment did not increase DA levels in NAc shell of knockout mice lacking the CB1 receptor (Fig. 2B).

3.2 *EX VIVO* ELECTROPHYSIOLOGICAL STUDIES

3.2.1 Effect of JWH-018 on inhibitory afferents to VTA dopaminergic cells

CB1-induced changes in extracellular DA levels in terminal regions critically depend upon VTA DA neuron spontaneous activity (Gessa et al. 1998; Diana et al., 1998), which mainly results from activation of CB1 receptors located on afferents arising from the Rostromedial Tegmental Nucleus (RMTg) and impinging onto VTA DA cells (Lecca et al., 2012; Melis et al., 2014). Therefore, we investigated whether or not JWH-018 might affect inhibitory synaptic transmission presumably arising from the RMTg onto VTA DA cells, by recording inhibitory postsynaptic currents (IPSCs), in a

whole-cell configuration, from lateral posterior VTA. Fig. 3A shows that bath application of JWH-018, at a concentration of 0.3 μM (5 min), significantly reduced IPSCs (by ~50 %, N= 7; one-way ANOVA + Dunnett's test, $F_{(18,108)}$ = 21.85, p < 0.0001). Fig. 3B shows that the effect of JWH-018 was accompanied by an increased paired-pulse ratio (from IPSC2/ IPSC1= 0.69 ± 0.1 to IPSC2/ IPSC1= 1.66 ± 0.3 before and after JWH-018, respectively; N = 7; two-tailed paired *t*-test, t = 2.08, p < 0.05). Fig. 3A also shows that JWH-018 effects on IPSCs required activation of CB1 receptors, since it was blocked in the presence of the CB1 receptor antagonist Rimonabant (SR141716A, 1 μM; two-way ANOVA, $F_{(1,190)}$ =31.51, *P*=0.0002, N=5). The decrease of $GABA_A$ IPSCs produced by JWH-018 was concentration dependent over the concentration range 100 nM to 1 μM (Fig. 4C, N= 7). In addition, its effect was mimicked by the structurally dissimilar CB1 agonist WIN 55,212-2 (300 nM- 3 μM, *n*= 6; IPSCs: one-way ANOVA, $F_{2,10}$= 20.68, p = 0.0003). The statistical analysis revealed that JWH-018 was more potent than WIN in reducing $GABA_A$ IPSCs (one-way ANOVA + Bonferroni's test; 300 nM: p < 0.05, t=3.68; 1 μM: p < 0.05, t=3.57; Fig. 4C). In addition, the following IC50 value have been observed, for WIN 55,212-2: between 0.9914 to 2.661 and for JWH-018: between 0.2039 to 0.3804; (Fig. 3C).

3.3 SCREENING FOR CANNABINOID-LIKE EFFECTS

3.3.1 Effect of JWH-018 on hypothermia and analgesia

Fig. 4 shows that systemic administration of JWH-018 (0.125-3.0 mg/kg i.p.) induces hypothermia (Fig. 4A), two-way ANOVA showed a significant effect of drug [$F_{(5,216)}$ = 43.83; p < 0.0001], time [$F_{(3,216)}$=24.51; p<0.0001] and time x drug interaction [$F_{(15,216)}$=7.906; p<0.0001] and increases the threshold for acute thermal nociception (Fig. 4C), two-way ANOVA showed significant effect of drug [$F_{(5,216)}$=15.81; p<0.0001], time [$F_{(3,216)}$=30.03; p<0.0001] and time x drug interaction [$F_{(15,216)}$=7.267;

p<0.0001] in rats. Changes of both body temperature and tail withdrawal were evaluated, respectively, at 20-70-120 min, and at 30-80-130 min after JWH-018 administration. AM 251 (3 mg/kg, i.p., 30 min before JWH-018) prevents hypothermia (Fig. 4B), two-way ANOVA showed significant effect of agonist drug [$F_{(3,112)}$=70.26;p<0.0001], time [$F_{(3,112)}$=13.37;, p<0.0001], interaction [$F_{(9,112)}$=15.91; p<0.0001] and the anti-nociceptive effect (Fig 4D: two-way ANOVA showed significant effect of agonist drug [$F_{(3,112)}$=14.86; p<0.0001], time [$F_{(3,112)}$=2.86; p<0.05], interaction [$F_{(9,112)}$=3.955; p < 0.0002] caused by JWH-018 (1 mg/kg i.p.).

3.3.2 Effect of JWH-018 on catalepsy and hypomotility

Fig. 4 also shows that JWH-018 (0.125-3 mg/kg i.p.) impairs motor skills in rats, causing an increase in the time spent on bars (Fig. 4E): two-way ANOVA showed a significant effect of drug [$F_{(5,216)}$=430.8; p<0.0001], time [$F_{(3,216)}$=183; p<0.0001] and time x drug interaction [$F_{(15,216)}$=48.52; p<0.0001]; and a reduction in time spent on the rotarod (Fig. 4G): two-way ANOVA showed a significant effect of drug [$F_{(5,216)}$=7.293; p<0.0001], time [$F_{(3,216)}$=8.985; p<0.0001] and time x drug interaction [$F_{(15,216)}$=1.829; p<0.05]. Motor effects were assessed at 40-90-140 min in the bar test and at 70-120-170 min in the rotarod test after JWH-018 administration. AM 251 (3 mg/kg, i.p., 30 min before JWH-018) prevents the effects induced by JWH-018 (1 mg/kg) in the bar (Fig. 4F): significant effect of agonist drug [$F_{(3,112)}$=43.08; p<0.0001], time [$F_{(3,112)}$=6.740; p<0.005], interaction [$F_{(9,112)}$=3.849; p<0.005] and in the rotarod (Fig 5F): significant effect of agonist drug [$F_{(3,112)}$=12.38; p<0.0001], time [$F_{(3,112)}$=3.566; p<0.0001], interaction [$F_{(9,112)}$=3.422; p=<0.0001] tests.

3.4 INTRAVENOUS SELF-ADMINISTRATION STUDIES

3.4.1 Acquisition, extinction, and reacquisition of JWH-018 self-administration in male Sprague-Dawley rats and involvement of CB1 cannabinoid receptors in this behaviour

In this experiment, acquisition, extinction, and reacquisition of JWH-018 self-administration (SA) of rats were studied. Fig. 5A shows that rats implanted with a jugular catheter were trained to self-administer JWH-018 (10 µg/kg/12 µl or 20 µg/kg/12 µl infusion) in single daily 1h session, under an initial Fixed Ratio (FR) 1 schedule, than increased to FR3. Fig. 5A also shows the average number of active and inactive nose-pokes performed by rats trained on JWH-018 SA during acquisition, extinction and reacquisition phases. Two-way ANOVA analysis of active/inactive nose pokes during acquisition, session with Rimonabant (SR-141716A) pre-administration, and extinction phases (sessions 1 to 47, N of rats = 14) showed a significant effect of nose-pokes [$F_{(1,26)}$= 55.51; p<0.0001), of session [$F_{(46,1196)}$= 4.8; p < 0.0001] and a significant nose-pokes x session interaction [$F_{(46,1196)}$= 8.93; p < 0.0001]. LSD *post-hoc* test showed significant differences between active *vs* inactive nose-pokes at the 16th, 21st, 23rd to 28th and from the 30th to the 47th sessions. Two-way ANOVA analysis of the reacquisition phase, applied to the 48th to the 54th session (N of rats = 6), showed a main effect of active *vs* inactive nose-pokes [$F_{(1,10)}$= 21.53; p < 0.001]. LSD *post-hoc* test showed significant differences between active *vs* inactive nose-pokes in all the sessions during the reacquisition phase. No differences were observed in active nose-poking on each Monday, following the weekend abstinence, compared with the last session of the preceding week. The percentage of rats that acquired JWH-018 self-administration was 90%.

Fig. 5B shows that rats failed to acquire vehicle self-administration (12 µl infusion) in single daily 1h session, under an initial Fixed Ratio (FR) 1 schedule, than

increased to FR3. Two-way ANOVA analysis did not show significant differences between active and inactive nose-pokes.

3.4.2 Acquisition, extinction, and reacquisition of JWH-018 self-administration in male C57B/6 mice

In this experiment, acquisition, extinction, and reacquisition of JWH-018 self-administration (SA) of mice were studied. Fig. 5B shows that mice implanted with a jugular catheter were trained to self-administer JWH-018 (15 µg/kg/25 µl or 30µg/kg/25 µl infusion) in single daily 2 hs session, under an initial Fixed Ratio (FR) 1 schedule, than increased to progressive ratio PR. Fig. 5B also shows the average number of active and inactive lever-presses performed by mice trained on JWH-018 SA during acquisition, extinction and reacquisition phases. Two-way ANOVA analysis of lever-presses during acquisition, session with PR and extinction phases (sessions 1 to 28, N of mice = 8) showed a significant effect of session [$F_{(27,270)}$=5.2; $p < 0.001$]). LSD *post-hoc* test showed significant differences between active *vs* inactive lever-presses at the 12th and 16th session. Two-way ANOVA analysis of the reacquisition phase, applied to the 29th to the 44th session (N of mice = 8), showed a main effect of sessions [$F_{(1,4)}$= 9.2; $p < 0.05$]. LSD *post-hoc* test showed significant differences between active *vs* inactive nose-pokes in at the 42th and 43rd sessions. The percentage of mice that acquired JWH-018 self-administration was 90%.

4.0DISCUSSION

In the present study, we show that a synthetic cannabinoid component of “Spice”, JWH-018, stimulates DA transmission preferentially in the NAc shell as compared to the NAc core and mPFC, and decreases $GABA_A$-mediated post-synaptic currents in VTA DA neurons through the activation of CB1 receptors. In addition, we observed that JWH-018 induces hypothermia, increases the threshold for acute thermal

nociception and impairs motor skills in rats in a dose-dependent manner. Moreover, JWH-018 serves as a reinforcer in a self-administration paradigm in rats and mice.

The ability of a substance to increase DA transmission preferentially in the NAc shell is common to drugs with abuse potential (Di Chiara et al. 2004; Di Chiara and Bassareo, 2007). JWH-018 increases DA transmission selectively in the NAc shell similarly to Δ^9-THC itself and the synthetic cannabinoid agonist WIN 55,212-2 (Tanda et al., 1997; Lecca et al., 2006). At the dose of 0.25 mg/kg, the maximal increase of DA in the NAc shell was about 65% over basal value, peaking at 20-60 min after JWH-018 and remaining at about 45% for the additional 60 min. Remarkably, DA transmission was not stimulated in the shell after administration of a higher dose (0.5 mg/kg i.p.) (Fig.1). Thus, the dose response curve of the effect of JWH-018 on dialysate DA had an inverted U-shape. This suggests that a narrow range of JWH-018 concentration activate DA release in the NAc shell. This biphasic dose-response curve might be due to formation of active metabolites of JWH-018, produced by phase I metabolism, that can readily cross the blood-brain-barrier and act as partial agonists or antagonists, thus inhibiting the effect of the parent drug (Dhawan et al., 2006; Wiebelhaus et al., 2012). Indeed, some hydroxylated metabolites of JWH-018 retain significant *in vitro* and *in vivo* activity (Brents et al., 2011). A glucuronidated metabolite (i.e. JWH-018-N-(5-hydroxypentyl) β-D-glucuronide) is an antagonist at CB1 receptors (Seely et al., 2012), but it is unlikely that this metabolite crosses the blood-brain-barrier. On the other hand, some studies suggest the involvement of pre-synaptic CB2 receptors located on DAergic terminals in the NAc (Xi et al., 2011; Morales and Bonci, 2012). JWH-018-induced increase of dialysate DA in the NAc shell of rats was completely prevented by pretreatment with CB1 receptors inverse agonists/antagonists Rimonabant and AM 251 (Fig. 2A), and was absent in mice with

genetic deletion of CB1 receptors (Fig. 2B). Notably, the dose of JWH-018 (0.25 mg/kg i.p.) able to stimulate NAc shell DA transmission was one-fourth the dose of Δ^9-THC (1 mg/kg i.p.) (De Luca et al., 2012). This is consistent with the higher affinity of JWH-018 for CB1 receptors, and with the IC50 for inhibition of GABA-A receptor mediated transmission over VTA DA neurons *ex vivo* produced by JWH-018 (0.3 μM) compared with WIN 55,212-2 (1μM) (see below).

It has already been established that administration of Δ^9-THC and other more potent cannabinoid agonists (e.g. WIN55, 212-2, HU-210, and CP55940) increases DA neuron activity (i.e. firing rates) *in vivo* in both anesthetized and non-anesthetized rats (French et al., 1997; Gessa et al., 1998) and in slice preparations containing the VTA (Cheer et al., 2000). This effect does not depend on direct activation of DA neurons in the VTA, but is mediated by the activation of CB1 receptors located on presynaptic GABAergic terminals onto VTA DA neurons (Matyas et al., 2008; Melis et al., 2014). Indeed, CB1 receptors are located on about 40% of RMTg afferents impinging upon VTA DA cells (Melis et al., 2014), and cannabinoids strongly reduce the inhibition of these extrinsic GABA afferents (Lecca et al., 2012). Given that, the observation that JWH-018 decreases inhibitory synaptic transmission presumably arising from the RMTg onto VTA DA cells is consistent with its pharmacological profile. Although we cannot identify definitively the sources of inhibitory afferents, we can assume that most of the caudal inputs electrically stimulated in our preparation are presumably originating from the RMTg (Matsui and Williams, 2011), given that it is one of the main caudally located inhibitory sources to the VTA. The effect of JWH-018 was not reversible on washout for 10 min, probably because this highly lipophilic drug is not readily washed out from the brain slice. Indeed, most likely due to the strong lipophilicity of cannabinoid drugs, their effects in brain slices are usually not reversible

(Ameri, 1999; Melis et al., 2004; Lecca et al., 2012). The effect of JWH-018 was accompanied by an increased paired-pulse ratio (Fig. 3B). Since changes in transmitter release have been reported to generally affect the paired-pulse ratio in several brain regions, including the VTA (Bonci and Williams, 1997; Melis et al., 2002; Melis et al., 2009), these results suggest that JWH-018 might reduce GABA release at a pre-synaptic site in agreement with CB1 receptor location (Melis et al., 2014).

It has been reported that high doses or chronic exposure to "Spice" produce severe medical consequences, including psychosis, violent behaviours, tachycardia, hyperthermia, and even death (Baumann et al., 2014). In order to investigate if the range of doses of JWH-018 that are able to stimulate DA transmission preferentially in the NAc shell could also induce similar behavioural effects as other natural or synthetic cannabinoids, we used the "tetrad" paradigm for screening the cannabinoid-like effects in a wide range of doses (0.125-3 mg/kg i.p.). We observed that injection of JWH-018 induces dose-dependent changes in body temperature, nociception and motor activity (Fig. 4). In particular, JWH-018 produced hypothermia that lasted at least 2h post injection, at least for the highest dose. This is consistent with the well-established effects of Δ^9-THC and synthetic cannabinoids on body temperature (Wiley et al., 2012,2014; Vigolo et al., 2015). JWH-018 also induced dose-dependent analgesia, as revealed by the increased latency of the animal to avoid a noxious stimulus. Finally, the drug induced catalepsy and impaired motor function in the rat as shown by the bar and rotarod tests. These data are also consistent with previous studies on cannabinoids (Martin et al., 1996; Wiley et al., 2012; Vigolo et al., 2015). Noteworthy, JWH-018 facilitates DArgic transmission at a dose that does not change the behavioural parameters in the tetrad. This is suggestive of the fact that the possible rewarding effect induced by JWH-018 (supported by the increase of DA in the NAc Shell) appears at

lower doses that do not produce significant behavioural alterations such as those highlighted in the tetrad.

In the present study, we also demonstrate that JWH-018 is self-administered by rats (Fig. 5) and mice (Fig. 6). First, rats consistently acquired operant behavior (nose-poking into an optical switch) that results in contingent intravenous infusions of JWH-018 (20 µg/kg/infusion) in single daily 1 h FR3 sessions. The reinforcing effects of JWH-018 in SA behavior were significantly reduced by the administration of the CB1 receptor inverse agonist/antagonist Rimonabant, suggesting that these effects of JWH-018 are mediated through CB1 receptors. Interestingly, after Rimonabant, the rate of responding for JWH-018 increased, suggestive of a rebound effect in the attempt to cope for the reduction of JWH-018 mediated reinforcement. Moreover, when JWH-018 was replaced by vehicle (38^{th} SA session, extinction phase), the responding rate as well as the number of injections did not decrease. The possibility that this behavior is unrelated to response-contingent training on JWH-018 is excluded by the fact that vehicle failed to induce responding (Fig. 5B). We suggest that the apparent resistance to extinction of instrumental responding after acquisition of JWH-018 SA is related to the acquisition of a habit modality, consistent with a role of cannabinoids in the habit learning (Hilario et al., 2007; Goodman and Packard, 2015). In this case it is likely that contextual cues instrumentally conditioned to JWH-018 were sufficient to maintain responding (Fig. 5A). Notably, when the vehicle was replaced once again with JWH-018, SA behavior immediately recovered (reacquisition phase) (Fig. 5A). Additionally, we found that C57BL/6 mice acquire operant behavior (lever-pressing) that results in intravenous JWH-018 infusion (30 µg/kg/infusion, FR1) and that their behavior increases under progressive-ratio (PR) schedule of reinforcement, showing that responding is specifically directed at obtaining the drug. Similarly to the rats' nose-

poking behavior, in mice, active lever-pressing did not decrease during extinction phase, in spite of the absence of drug associated cues. Importantly, when the vehicle was replaced once again with JWH-018, SA behavior immediately recovered (reacquisition phase) and the inactive lever-presses decreased (Fig. 6), showing that the animals' behavior was specifically modulated by the drug infusion.

These observations suggest that prolonged use of JWH-018 may induce a withdrawal syndrome related to significant alterations in emotional processing, cognitive functioning and disruption in affective states, as already observed in humans (Zimmermann et al., 2009). JWH-018, similar or even stronger than THC, may disrupt cortical processes responsible for context updating and the automatic orientation of attention (D'Souza et al., 2012). Indeed, cannabinoids modulate prefrontal cortex activity by increasing extracellular glutamate and DA levels and decreasing the release of GABA (Ferraro et al., 2001; Pistis et al., 2002). Further studies aimed at investigate the nature and persistence of JWH-018 dependence, are in progress.

In conclusion, we have shown that JWH-018 shares with the active principle of Cannabis, Δ^9-THC, the property of stimulating DA transmission *in vivo* in a specific DA terminal area, the NAc shell, most likely, by reducing $GABA_A$-receptor mediated inhibition of DA neuronal activity in the VTA. Moreover, JWH-018 shares with Δ^9-THC its main behavioural properties. These properties are predictive of JWH-018 reinforcing properties and abuse liability as suggested by the ability of JWH-018 to be self-administered by rats and mice.

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Figure legends

Fig.1

Effect of JWH-018 administration on DA transmission in the NAc shell, NAc core, and mPFC

Results are expressed as mean ± SEM of change in DA extracellular levels expressed as the percentage of basal values. The arrow indicates the start of JWH-018 i.p. injection at the dose of 0.125 mg/kg (*diamonds*), 0.25 mg/kg (*squares*), 0.5 mg/kg (*triangles*) or vehicle (*circles*) in the NAc shell (A), NAc core (B), and mPFC (C). Solid symbol: $p < 0.05$ with respect to basal values; *$p < 0.05$ vs veh NAc shell group; § $p < 0.05$ vs 0.25 NAc core group; × $p < 0.05$ vs 0.25 mPFC group; × $p < 0.05$ vs 0.5 NAc shell group (NAc shell N= 32 ; NAc core N= 23; mPFC N= 29) (Three-way ANOVA, Tukey's HSD post hoc).

Fig.2

Blockade of JWH-018 effect on increase of DA transmission in the NAc shell by SR141716A and AM 251 and by genetic deletion

Results are expressed as mean ± SEM of change in DA extracellular levels expressed as the percentage of basal values. (A) The arrow indicates the start of JWH-018 i.p. injection at the dose of 0.25 mg/kg i.p. in rats pre-treated with Rimonabant (SR141716A, 1.0 mg/kg i.p., 30 min before agonist) (*diamonds*) or AM 251 (1.0 mg/kg i.p., 30 min before agonist) (*circles*) or vehicle (*squares*). Solid symbol: $p < 0.05$ with respect to basal values; *$p < 0.05$ vs veh group. (NAc shell veh N= 13 ; NAc shell SR N=7; NAc shell AM N= 3) (Two-way ANOVA, Tukey's HSD post hoc). (B) The arrow indicates the start of JWH-018 i.p. injection at the dose of 0.3 mg/kg in CB1 $^{+/+}$ (*squares*) and CB1 $^{-/-}$ (*circles*) mice. Solid symbol: $p < 0.05$ with respect to basal

values; *$p < 0.05$ vs CB1 $^{+/+}$ group. (NAc shell CB_1 $^{+/+}$ N= 32; NAc shell CB1 $^{-/-}$ N= 26) (Two-way ANOVA, Tukey's HSD post hoc).

Fig. 3

JWH-018 inhibits GABA synaptic transmission elicited by stimulation of caudal afferents in rat posterior VTA DA cells

(A) JWH-018 (0.3 μM, 5 min) reduces IPSCs amplitude (one-way ANOVA, $p < 0.0001$) through activation of CB1 receptors (two-way ANOVA, $p < 0.0001$). All data are normalized to the respective baseline (5 min of baseline). Black bar shows time of superfusion of JWH-018 in the presence (*open circles*) and absence (*grey circles*) of the CB1 receptor antagonist Rimonabant (SR141716A, 1 μM). SEM bars are smaller than symbols in some cases. Representative traces are shown in the inset. Scale bar: 100 pA, 15 ms. (B) JWH-018 enhances the paired-pulse ratio of $GABA_A$ IPSCs (two-tailed paired *t*-test; $p < 0.05$). The circles represent the paired-pulse ratio for each of the experiments in A before (basal) and after the application of JWH-018 (JWH-018), while the bars represent the averaged paired-pulse ratio. (C) Concentration-response relationship for percentage decrease in $GABA_A$ IPSCs amplitude produced by JWH-018 (0.1-1 μM) and WIN (0.3-3 μM). Each point shows the mean ± SEM of responses of different neurons (N= 6 and 7, respectively).

Fig.4

Systemic administration of JWH-018 (0.125-3 mg/kg ip) induces hypothermia, increases the threshold for acute thermal nociception, and impairs motor skills in rats

Changes of the body temperature (A) and of the tail withdrawal (C) were evaluated, respectively, at 20-70-120 min, and at 30-80-130 min after JWH-018 administration. The selective CB1 receptor antagonist AM 251 (3 mg/kg, i.p., 30 min before JWH-

018) prevents hypothermia (B) and the anti-nociceptive effect (D), caused by JWH-018 (1 mg/kg ip). Data are expressed in absolute value (body temperature in C° and nociception threshold in seconds), and correspond to the mean ± SEM of 8-10 determinations per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs veh, # $p < 0.05$, ### $p < 0.001$ vs JWH-018 (1 mg/kg ip). (N=10) (Two-way ANOVA, Bonferroni's test post hoc). Motor effects were assessed at 40-90-140 min in the bar test (E) and at 70-120-170 min in the rotarod test (G) after JWH-018 administration. The selective CB1 receptor antagonist AM 251 (3 mg/kg, ip, 30 min before JWH-018) prevents the effects induced by JWH-018 (1 mg/kg) in the bar (F) and in the rotarod (H) tests. Data are expressed as absolute values (seconds) and correspond to the mean ± SEM of 8-10 determinations per group. * $p< 0.05$, *** $p< 0.001$ vs veh, ### $p< 0.001$ vs JWH-018 (1 mg/kg ip). (N= 10) (Two-way ANOVA, Bonferroni's test post hoc).

Fig. 5

JWH-018 self-administration in rats

(A) JWH-018 self-administration by Sprague Dawley rats and involvement of CB1 cannabinoid receptors in this behaviour. Number of active nose pokes (*circles*) that resulted in JWH-018 infusion (10 or 20 µg/kg/infusion) or inactive ones (*triangles*) during each 1-h daily session under FR1 and FR 3 during acquisition (sessions 1 to 37), extinction (sessions 38 to 47) and reacquisition (sessions 48 to 54) phases. On sessions 28th and 29th the effect of Rimonabant (SR141716A) on the JWH-018 SA was tested. Results are expressed as mean ± SEM (N sessions 1-47 = 14, sessions 48-54 = 6) *p<0.05 vs inactive nose pokes; ANOVA followed by LSD *post hoc* test. (B) Number of active nose pokes (*circles*) that resulted in Vehicle infusion (12 µl infusion) or inactive ones (*triangles*) during each 1-h daily session under FR1 and FR 3 during

acquisition (sessions 1 to 26). Results are expressed as mean ± SEM (N = 7); ANOVA followed by LSD *post hoc* test.

Fig. 6

JWH-018 self-administration in mice

JWH-018 self-administration by C57BL/6 mice under fixed (FR1) and progressive (PR) reinforcement schedules. Number of active lever-presses (*circles*) that resulted in JWH-018 infusion (15 or 30 μg/kg/inf) or inactive lever-presses (*triangles*) during each 2 hs daily session under FR1 (1^{st}-15^{th} sessions), and PR (16^{th} session) reinforcement schedules. Results are expressed as mean ± SEM (N=8), *$p<0.05$ vs inactive lever-presses; ANOVA followed by LSD *post hoc* test.

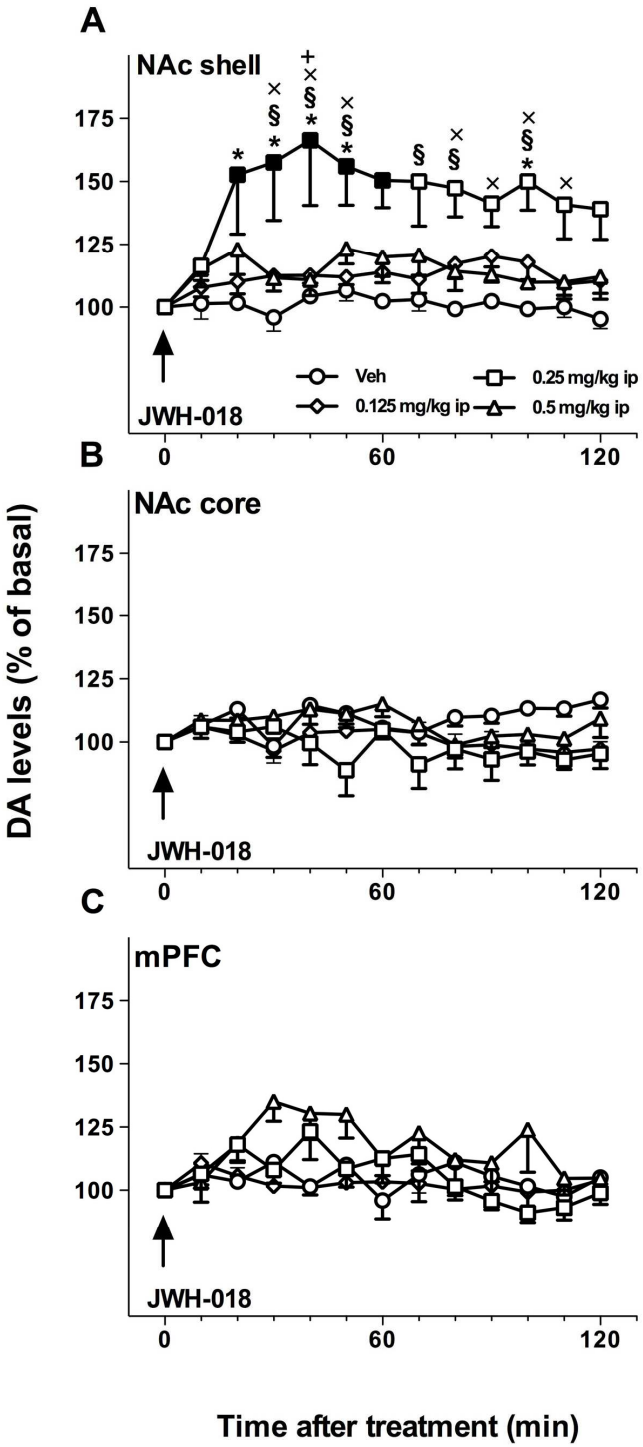

A
NAc shell
175
150
125
100
Veh
0.25 mg/kg ip
0.125 mg/kg ip
0.5 mg/kg ip
JWH-018
0
60
120
B
NAc core
175
150
125
100
JWH-018
0
60
120
C
mPFC
175
150
125
100
JWH-018
0
60
120
DA levels (% of basal)
Time after treatment (min)

Figure 1

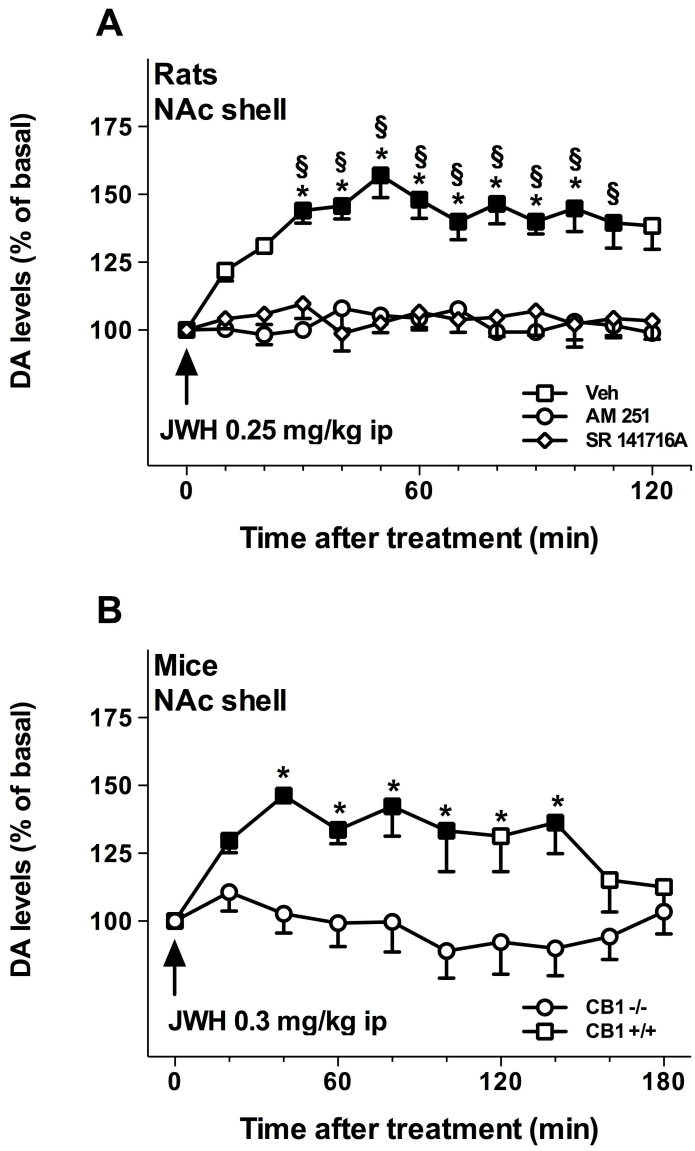

A
Rats
NAc shell
DA levels (% of basal)
175
150
125
100
JWH 0.25 mg/kg ip
Veh
AM 251
SR 141716A
0
60
120
Time after treatment (min)
B
Mice
NAc shell
DA levels (% of basal)
175
150
125
100
JWH 0.3 mg/kg ip
CB1 -/-
CB1 +/+
0
60
120
180
Time after treatment (min)

Figure 2

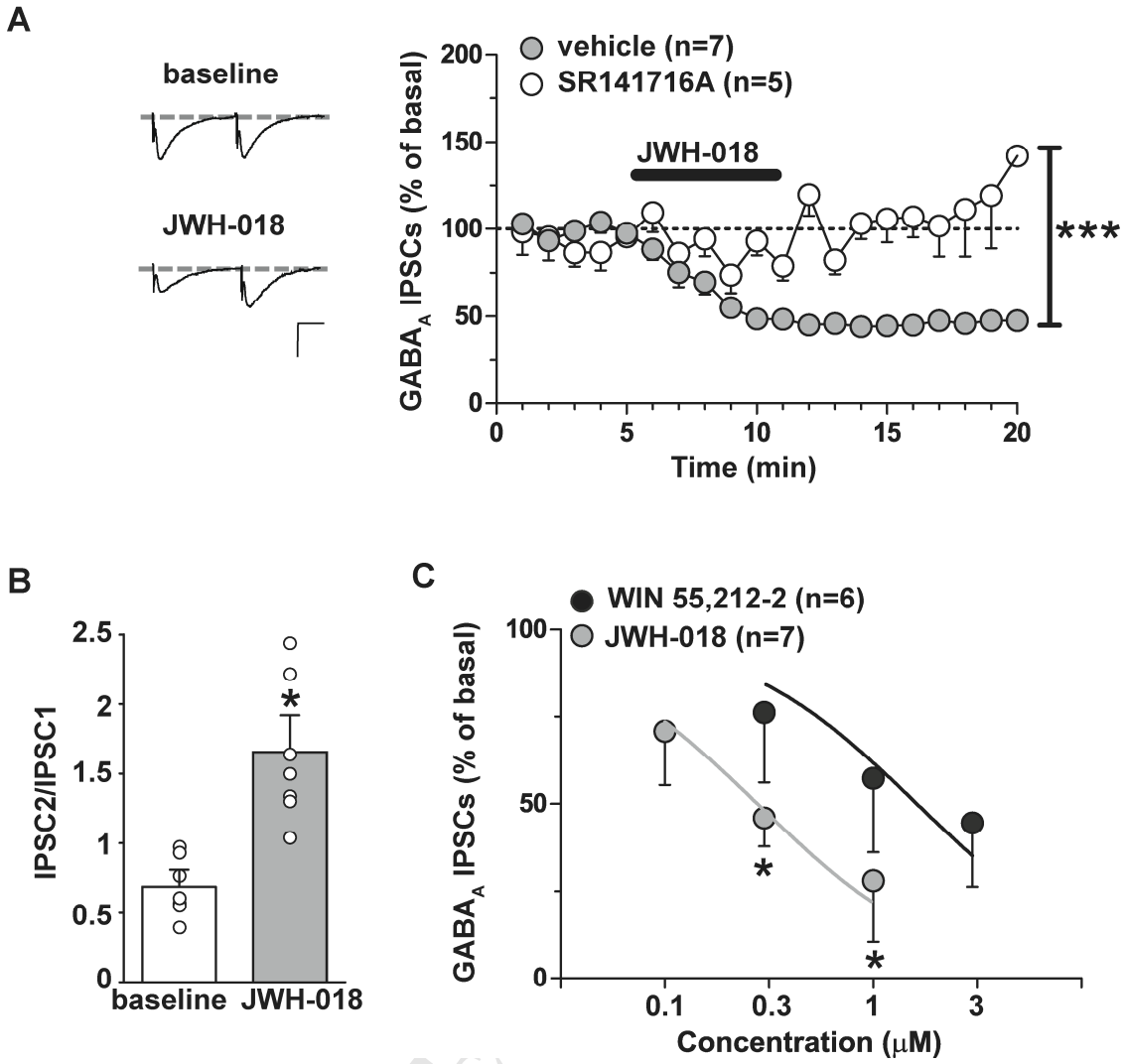


Figure 3

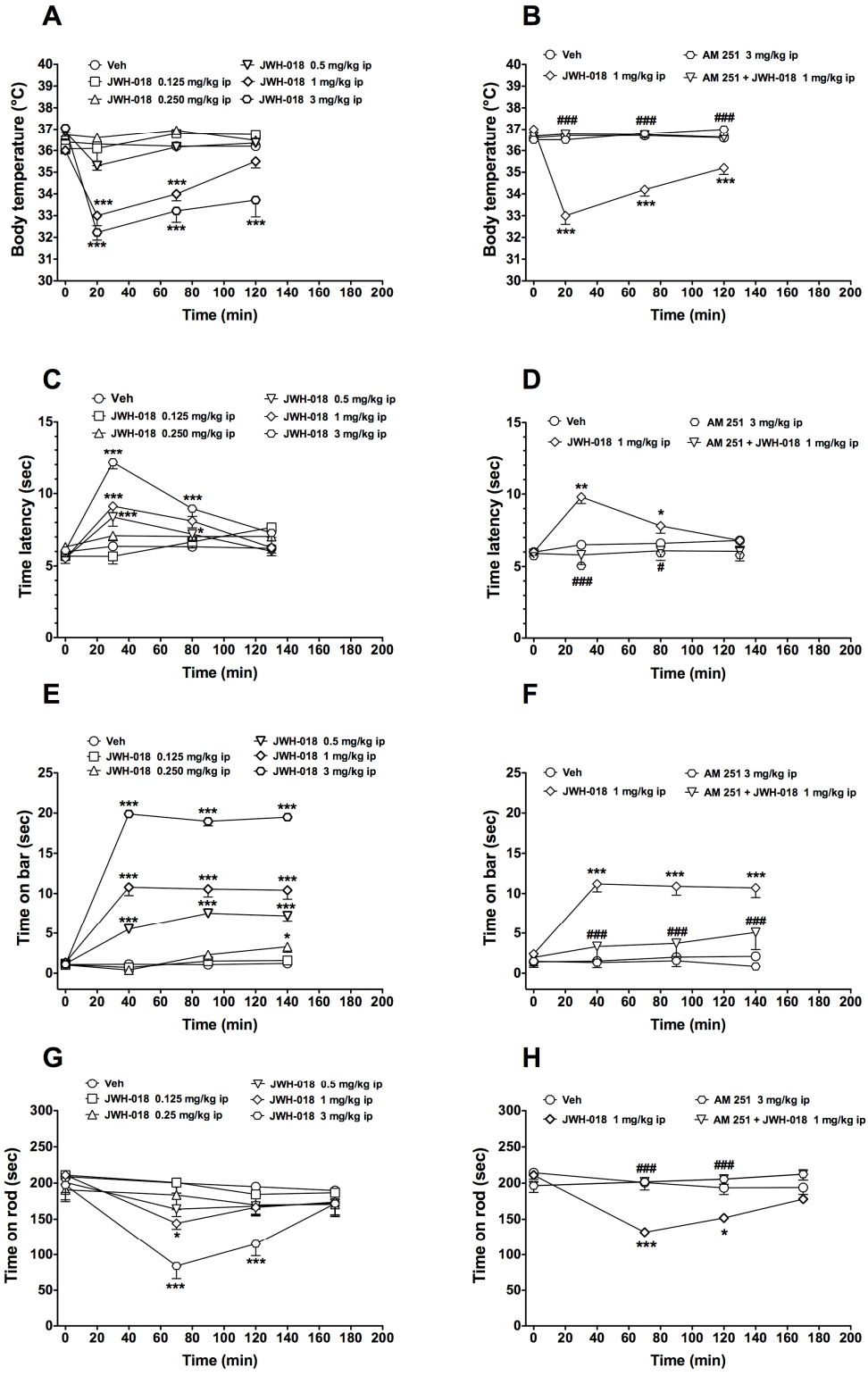

A
Veh
JWH-018 0.125 mg/kg ip
JWH-018 0.250 mg/kg ip
JWH-018 0.5 mg/kg ip
JWH-018 1 mg/kg ip
JWH-018 3 mg/kg ip
Body temperature (°C)
Time (min)
B
Veh
JWH-018 1 mg/kg ip
AM 251 3 mg/kg ip
AM 251 + JWH-018 1 mg/kg ip
Body temperature (°C)
Time (min)
C
Veh
JWH-018 0.125 mg/kg ip
JWH-018 0.250 mg/kg ip
JWH-018 0.5 mg/kg ip
JWH-018 1 mg/kg ip
JWH-018 3 mg/kg ip
Time latency (sec)
Time (min)
D
Veh
JWH-018 1 mg/kg ip
AM 251 3 mg/kg ip
AM 251 + JWH-018 1 mg/kg ip
Time latency (sec)
Time (min)
E
Veh
JWH-018 0.125 mg/kg ip
JWH-018 0.250 mg/kg ip
JWH-018 0.5 mg/kg ip
JWH-018 1 mg/kg ip
JWH-018 3 mg/kg ip
Time on bar (sec)
Time (min)
F
Veh
JWH-018 1 mg/kg ip
AM 251 3 mg/kg ip
AM 251 + JWH-018 1 mg/kg ip
Time on bar (sec)
Time (min)
G
Veh
JWH-018 0.125 mg/kg ip
JWH-018 0.25 mg/kg ip
JWH-018 0.5 mg/kg ip
JWH-018 1 mg/kg ip
JWH-018 3 mg/kg ip
Time on rod (sec)
Time (min)
H
Veh
JWH-018 1 mg/kg ip
AM 251 3 mg/kg ip
AM 251 + JWH-018 1 mg/kg ip
Time on rod (sec)
Time (min)

Figure 4

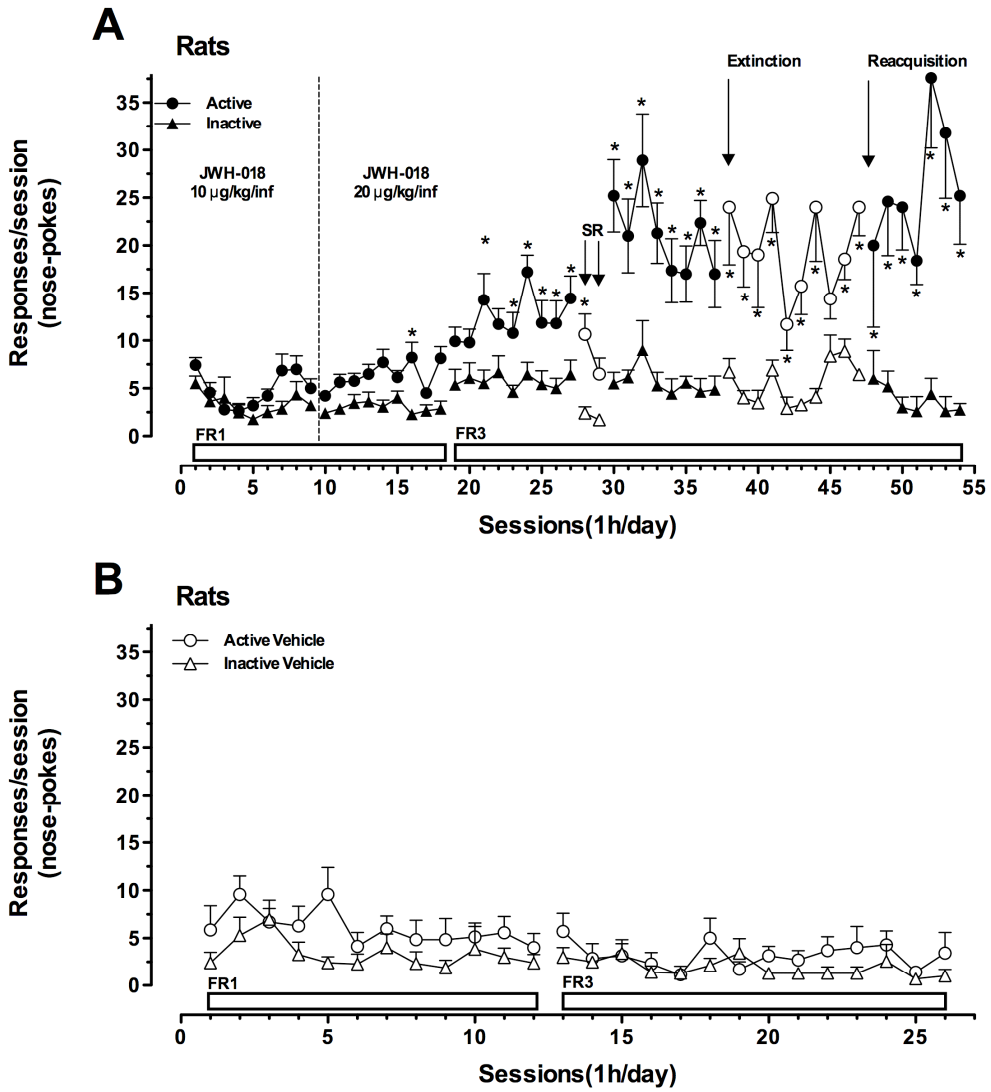

A
Rats
Active
Inactive
JWH-018
10 µg/kg/inf
JWH-018
20 µg/kg/inf
SR
Extinction
Reacquisition
FR1
FR3
Responses/session
(nose-pokes)
Sessions(1h/day)
B
Rats
Active Vehicle
Inactive Vehicle
FR1
FR3
Responses/session
(nose-pokes)
Sessions(1h/day)

Figure 5

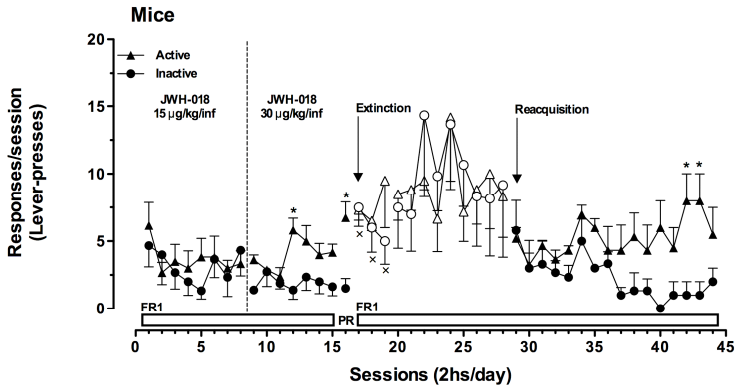
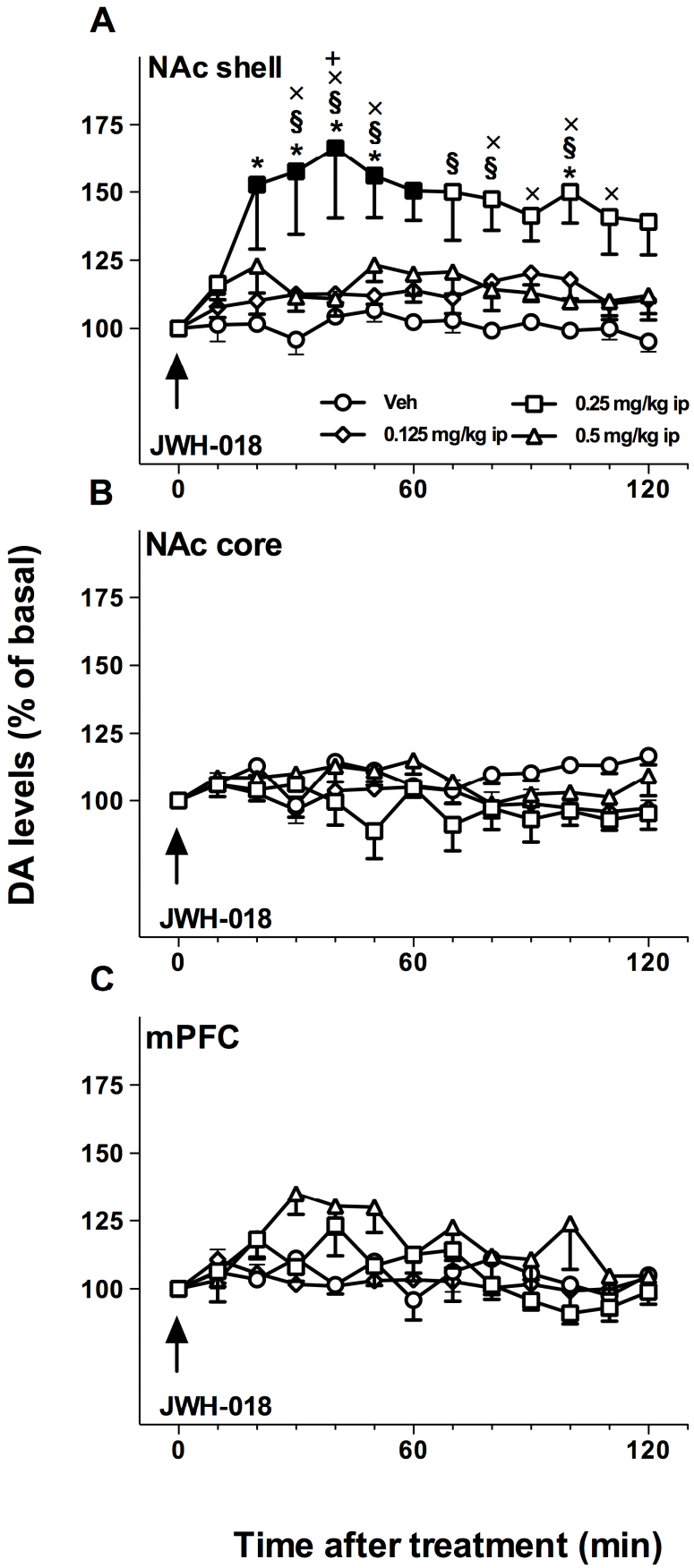
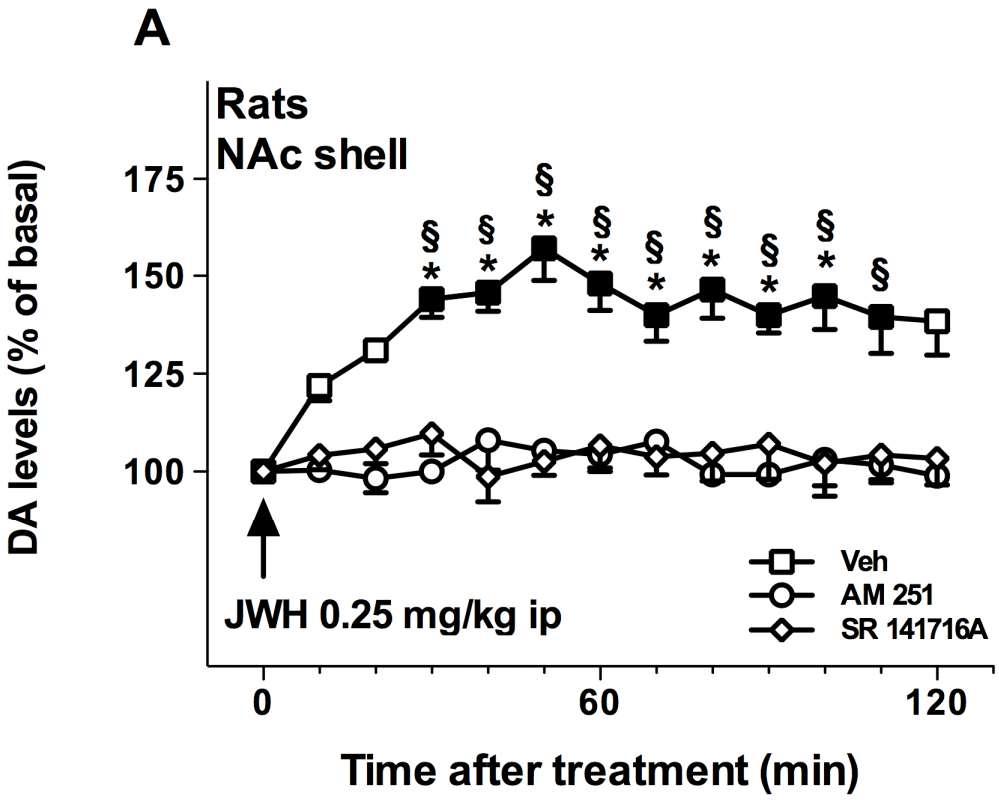
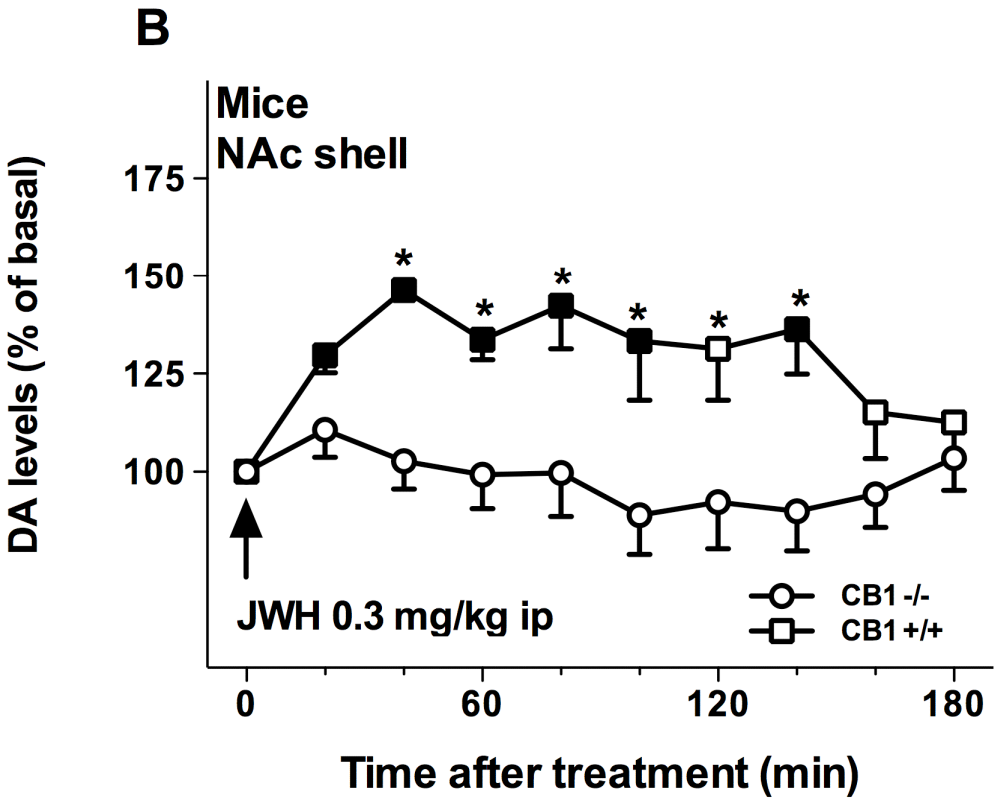
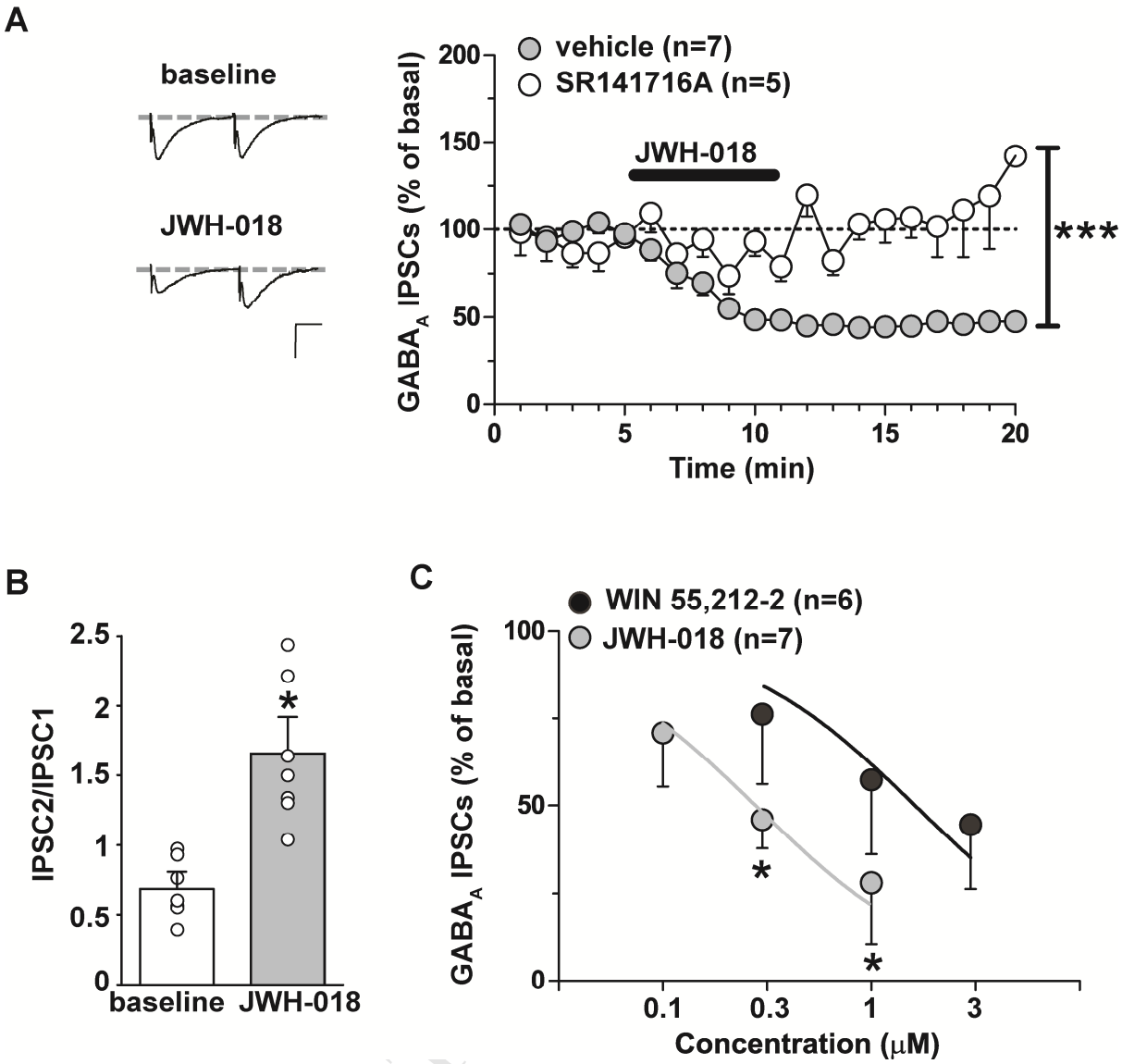


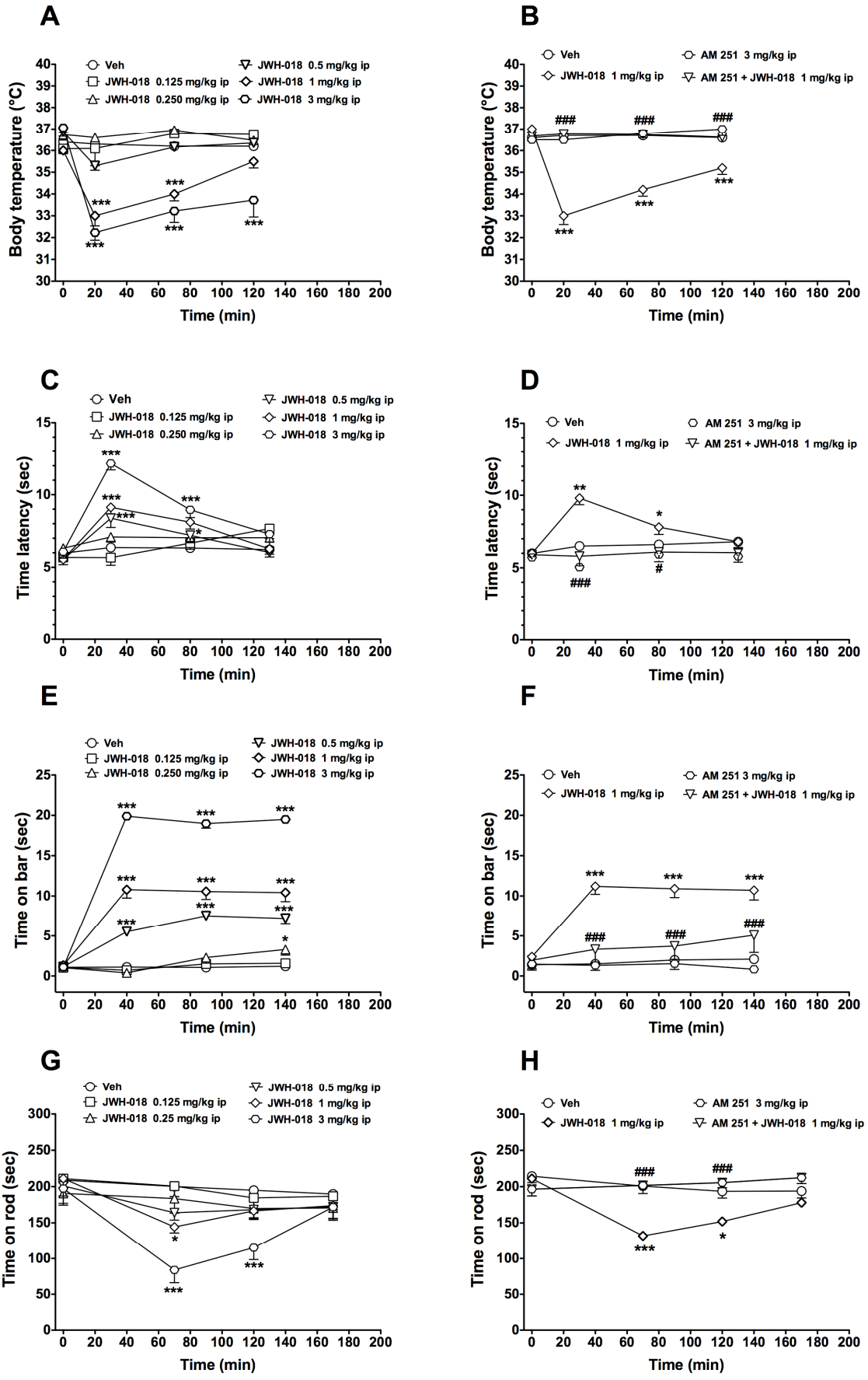
Figure 6

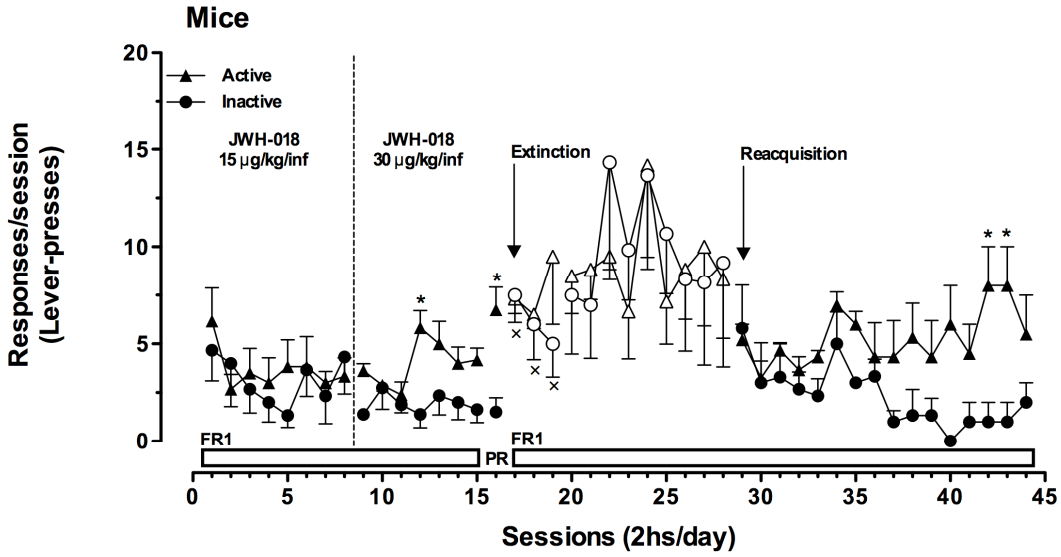

A
NAc shell
175
150
125
100
JWH-018
Veh
0.25 mg/kg ip
0.125 mg/kg ip
0.5 mg/kg ip
0
60
120
B
NAc core
175
150
125
100
JWH-018
0
60
120
C
mPFC
175
150
125
100
JWH-018
0
60
120
DA levels (% of basal)
Time after treatment (min)

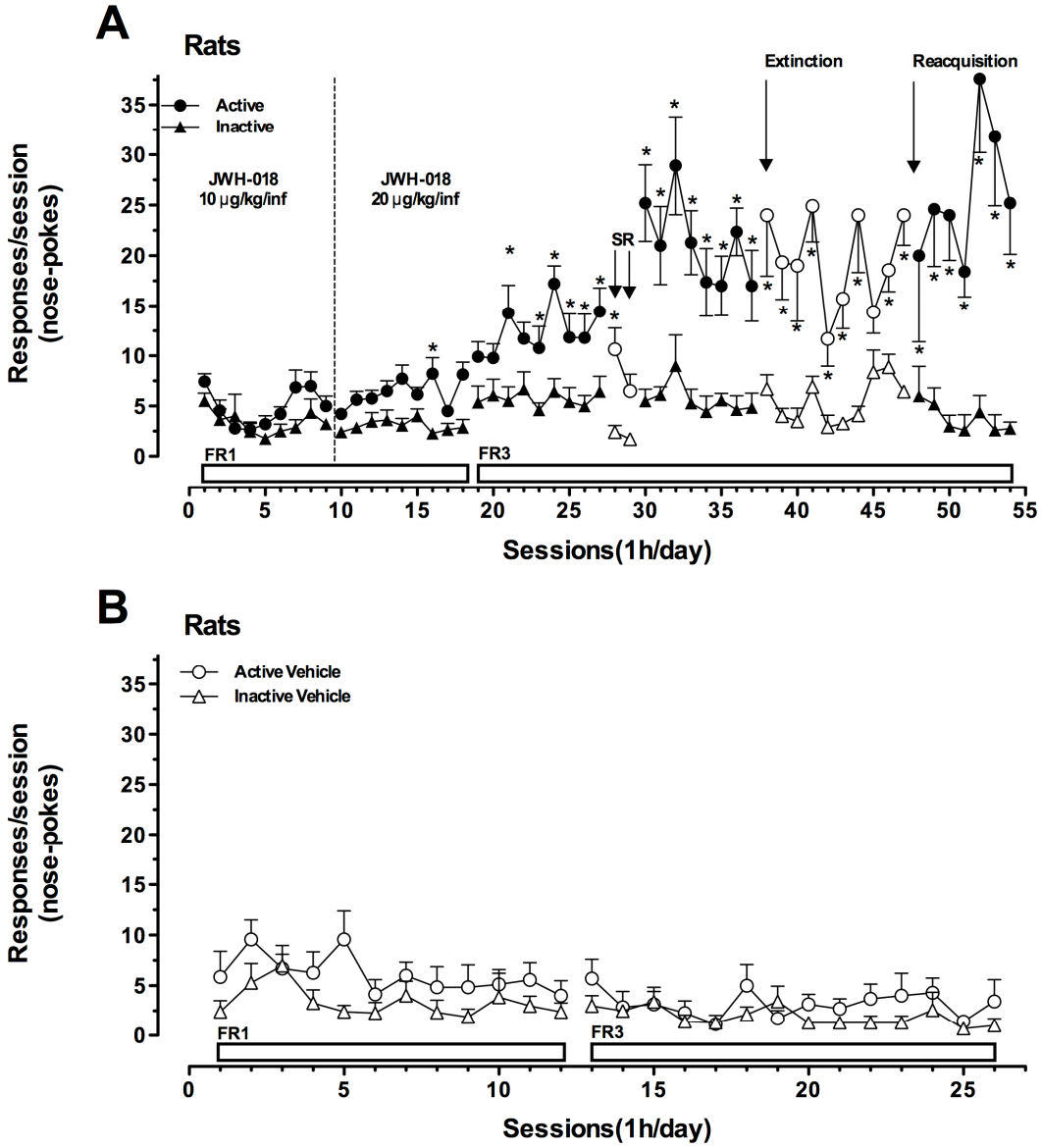

A
Rats
NAc shell
DA levels (% of basal)
175
150
125
100
JWH 0.25 mg/kg ip
Veh
AM 251
SR 141716A
0
60
120
Time after treatment (min)


B
Mice
NAc shell
DA levels (% of basal)
175
150
125
100
JWH 0.3 mg/kg ip
CB1 -/-
CB1 +/+
0
60
120
180
Time after treatment (min)


A
baseline
JWH-018
vehicle (n=7)
SR141716A (n=5)
JWH-018
GABA$_A$ IPSCs (% of basal)
200
150
100
50
0
***
0
5
10
15
20
Time (min)
B
IPSC2/IPSC1
2.5
2
1.5
1
0.5
0
*
baseline
JWH-018
C
WIN 55,212-2 (n=6)
JWH-018 (n=7)
GABA$_A$ IPSCs (% of basal)
100
50
0
*
*
0.1
0.3
1
3
Concentration (μM)


A
Veh
JWH-018 0.125 mg/kg ip
JWH-018 0.250 mg/kg ip
JWH-018 0.5 mg/kg ip
JWH-018 1 mg/kg ip
JWH-018 3 mg/kg ip
Body temperature (°C)
Time (min)
B
Veh
JWH-018 1 mg/kg ip
AM 251 3 mg/kg ip
AM 251 + JWH-018 1 mg/kg ip
Body temperature (°C)
Time (min)
C
Veh
JWH-018 0.125 mg/kg ip
JWH-018 0.250 mg/kg ip
JWH-018 0.5 mg/kg ip
JWH-018 1 mg/kg ip
JWH-018 3 mg/kg ip
Time latency (sec)
Time (min)
D
Veh
JWH-018 1 mg/kg ip
AM 251 3 mg/kg ip
AM 251 + JWH-018 1 mg/kg ip
Time latency (sec)
Time (min)
E
Veh
JWH-018 0.125 mg/kg ip
JWH-018 0.250 mg/kg ip
JWH-018 0.5 mg/kg ip
JWH-018 1 mg/kg ip
JWH-018 3 mg/kg ip
Time on bar (sec)
Time (min)
F
Veh
JWH-018 1 mg/kg ip
AM 251 3 mg/kg ip
AM 251 + JWH-018 1 mg/kg ip
Time on bar (sec)
Time (min)
G
Veh
JWH-018 0.125 mg/kg ip
JWH-018 0.25 mg/kg ip
JWH-018 0.5 mg/kg ip
JWH-018 1 mg/kg ip
JWH-018 3 mg/kg ip
Time on rod (sec)
Time (min)
H
Veh
JWH-018 1 mg/kg ip
AM 251 3 mg/kg ip
AM 251 + JWH-018 1 mg/kg ip
Time on rod (sec)
Time (min)




A
Rats
Active
Inactive
JWH-018
10 µg/kg/inf
JWH-018
20 µg/kg/inf
SR
Extinction
Reacquisition
FR1
FR3
Responses/session
(nose-pokes)
Sessions(1h/day)
B
Rats
Active Vehicle
Inactive Vehicle
FR1
FR3
Responses/session
(nose-pokes)
Sessions(1h/day)

HIGHLIGHTS

- JWH-018 is a full CB1 and CB2 agonist that has been frequently identified in Spice.
- JWH-018 increases NAc shell DA and decreases $GABA_A$-mediated IPSC in VTA DA neurons
- JWH-018 induces dose-dependent tetrad-like effects and IVSA in rats and mice
- Spice effects suggest a greater prevalence of dependence and risk to health than MJ

STIMULATION OF *IN VIVO* DOPAMINE TRANSMISSION AND INTRAVENOUS SELF-ADMINISTRATION IN RATS AND MICE BY JWH-018, A SPICE CANNABINOID

Supplemental Informations

Supplemental Materials and Methods

Animals

Male Sprague-Dawley rats (Harlan, Italy) weighing 275-300 g were used for *in vivo* microdialysis, cannabinoid-like effects screening experiments and IVSA studies. For additional IVSA studies, adult male mice C57BL/6 (obtained from The Scripps Research Institute breeding colony), aged 3-6 months , 25-30 g at the beginning of the experiments were used. Male Sprague-Dawley rats (Harlan, Italy) on postnatal day 14-21 were used for *ex vivo* electrophysiological studies. For additional microdialysis experiments, young adult (8-10 weeks) homozygous mutant mice ($CB1^{-/-}$) and wild type ($CB1^{+/+}$) littermates derived from heterozygous mattings were generated and genotyped as described previously (Marsicano et al., 2002). All animal experiments were carried out in accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research according to Italian (D.L. 116/92 and 152/06) and European Council directives (609/86 and 63/2010) and in compliance with the approved animal policies by the Ethical Committee for Animal Experiments (CESA, University of Cagliari) and the Italian Ministry of Health. We made all efforts to minimize pain and suffering, and to reduce the number of animals used. Animals were housed in groups of three to six in standard conditions of temperature (21 ± 1°C) and humidity (60%) under a 12h/12h light/dark cycle (lights on at 7.00 am) with food and water available *ad libitum.* Adult

male mice C57BL/6 obtained from The Scripps Research Institute breeding colony, and housed 2-4 mice/cage in Plexiglas cages 28x17x11.5 cm. Animals were then housed singly in these cages 1 week prior to beginning the intravenous self-administration experiments and throughout the experiments, in order to protect catheterized subjects. Colony temperature was maintained at 24°C. Subjects were provided with free access to food and maintained under conditions of a reverse light dark cycle: lights on at 22:00 hours, lights off at 10:00 hours.

Drugs

JWH-018 and WIN-55212 were purchased from Tocris (Bristol, UK). Rimonabant hydrochloride (SR141716A) and AM 251 were purchased from RD-Sigma (Italy). For *in vivo* microdialysis and cannabinoid-like effects screening studies JWH-018, SR 141716A and AM 251 were solubilized in 2% EtOH, 2% Tween 80 and 94 % saline and administered intraperitoneally (i.p.) at a different doses depending on the group of animals. JWH-018: 0.125- 3.0 mg/kg (3 ml/kg) for in vivo studies in rats; 0.3 mg/kg (10 ml/kg) for in vivo studies in mice. SR 141716A and AM 251: at doses of 1 and 3 mg/kg (3 ml/kg), 30 min prior to JWH-018. For *ex vivo* electrophysiological studies, JWH-018, WIN-55212 and SR 141716A were solubilized in DMSO (final concentration <0.001%).

***In vivo* microdialysis**

Surgery. Male Sprague-Dawley rats (275-300 g; Harlan, Italy) were anaesthetized with Equitesin (3ml/kg ip; chloral hydrate 2.1 g, sodium pentobarbital 0.46 g, $MgSO_4$ 1.06 g, propylene glycol 21.4 ml, ethanol (90%) 5.7 ml, H_2O 3 ml) and implanted with vertical dialysis probes (1.5 or 3 mm dialyzing portion for NAc or mPFC, respectively) 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). For additional microdialysis experiments, young mice weighing between 25-30 g were anaesthetized (Sodium Penthobarbital, 50 mg/kg i.p.; Sigma-Aldrich, Italy) and implanted with vertical dialysis probes (1 mm dialyzing portion) in the NAc shell (A+1.4, L 0.4 from bregma, V-4.8 from dura). In all cases, the experimenter was blind to the genotype.

Analytical Procedure. On the day following surgery, probes were perfused with Ringer's solution (147 mM NaCl, 4 mM KCl, 2.2 mM $CaCl_2$) at a constant rate of 1 µl/min. Dialysate samples (10 or 20 µl) 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. 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: 50 mM NaH_2PO_4, 0.1 mM Na_2-EDTA, 0.5 mM n-octyl sodium sulfate, 15% (v/v) methanol, pH 5.5. The sensitivity of the assay for DA was 5 fmol/sample.

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.

Whole cell patch clamp recordings *ex vivo*

The preparation of VTA slices was as described previously (Melis et al., 2014). Briefly, male Sprague Dawley rats (14-21 d, Harlan, Italy), were anesthetized with isoflurane and euthanized by guillotine. A block of tissue containing the midbrain was rapidly dissected

and sliced in the horizontal plane (300 and 230 µm for rat and mouse slices, respectively) with a vibratome (Leica) in ice-cold low-Ca^{2+} solution containing (in mM): 126 NaCl, 1.6 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 0.625 $CaCl_2$, 18 $NaHCO_3$, and 11 glucose. Slices were transferred to a holding chamber with artificial cerebrospinal fluid (ACSF, 37°C) saturated with 95% O_2 and 5% CO_2 containing (in mM): 126 NaCl, 1.6 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 18 $NaHCO_3$, and 11 glucose. Slices were allowed to recover for at least 1 h before being placed, as hemislices, in the recording chamber and superfused with the ACSF (36°C) saturated with 95% O_2 and 5% CO_2. Cells were visualized with an upright microscope with infrared illumination (Axioskop FS 2 plus, Zeiss), and whole-cell patch clamp recordings were made by using an Axopatch 200B amplifier (Molecular Devices, CA). Whole cell voltage-clamp recordings were made with electrodes filled with a solution containing the following (in mM): 144 KCl, 10 HEPES, 3.45 BAPTA, 1 $CaCl_2$, 2.5 Mg_2ATP, and 0.25 Mg_2GTP (pH 7.2-7.4, 275-285 mOsm). Experiments were begun only after series resistance had stabilized (typically 10-30 M□). Series and input resistance were monitored continuously on-line with a 5 mV depolarizing step (25 ms). Data were filtered at 2 kHz, digitized at 10 kHz, and collected on-line with acquisition software (pClamp 8.2, Axon Instruments, CA). DA neurons from the posterior lateral VTA were identified according to the already published criteria (Melis et al., 2014): cell morphology and anatomical location (i.e. medial to the medial terminal nucleus of the accessory optic tract), slow pacemaker-like firing rate (<5 Hz), long action potential duration (>2 ms), and the presence of a large I_h current (≥100 pA) (Johnson and North, 1992) that was assayed immediately after break-in, using 13 incremental 10 mV hyperpolarizing steps (250 ms) from a holding potential of -70 mV. A

bipolar stainless steel stimulating electrode (FHC, USA) was placed ~500 μm caudal to the recording electrode and was used to stimulate at a frequency of 0.1 Hz. Paired stimuli were given with an interstimulus interval of 50 ms, and the ratio between the second and the first IPSCs (IPSC2/IPSC1) was calculated and averaged for a 5 min baseline (Melis et al., 2002).

Screening for cannabinoid-like effects *in vivo*

Tests were performed after i.p. injection of vehicle, JWH-018 and AM 251. Tests were performed at different times post-injection depending on the effect under investigation. For body temperature, at 20, 70 and 120 min post injection; for nociception at 30, 80 and 130 min; for motor activity, bar test was performed at 40, 90 and 140 min and rotarod test at 70,120 and 170 min.

Body temperature. Core temperature was evaluated by a probe (1 mm diameter) gently inserted, after lubrication with liquid vaseline, into the rectum of the rat (to about 2 cm) and left in position until stabilization of temperature (about 10 sec; adapted from Vigolo et al., 2015).

Thermal pain. Acute thermal nociception was evaluated using the tail withdrawal test (adapted from Vigolo et al., 2015). Rats were restrained in a dark plastic cylinder closed at both sides with plastic mesh, which allowed the rats to breathe normally. Then half of the tail was dipped in water of 48 °C and the latency (in seconds) until the tail was left in the water was recorded. A cut off of 15 seconds was set to avoid tissue damage. No signs of damage, burn or variation in rat tail sensitivity were observed after the repetition of three consecutive tests at 48 °C.

Motor activity. Changes in motor activity induced by JWH-018 were studied using a battery of behavioral tests validated to specifically assess different aspects of motor behavior in static (bar test) and dynamic conditions (rotarod test) (Marti et al., 2004, 2005).

Bar test: It measures the degree of akinesia/catalepsy, that is, the time the rat remained immobile once his forelimbs are placed on a wooden bar at three different heights (3, 6 and 9 cm). The time spent each paw on the bar is measured. The total time spent on the three tests was recorded (cut off, 20 sec).

Rotarod test: The test measures different motor parameters as the motor coordination, the locomotive ability (akinesia/bradykinesia), the balance ability, the muscular tone. The animals are placed on a rotating cylinder that increases velocity automatically in a constant manner (0-60 rotations/min in 5 min). The time spent on the cylinder is measured.

Intavenous self administration studies

Rats

Daily SA sessions were carried out in chambers housed in sound proof boxes (Coulbourn Instruments, Allentown, NJ, USA), provided with two nose-poke holes, one active and the other inactive. A yellow/green light was placed over the active hole and a red light over the inactive one as discriminative stimuli. Prior to each daily session, the jugular catheter was flushed with 0.1 ml of sterile saline and the rats were placed in the SA box.

Rats were anaesthetized with Equitesin (3 ml/kg intraperitoneal (ip); chloral hydrate 2.1 g, sodium pentobarbital 0.46 g, MgSO 1.06 g, propylene glycol 21.4 ml, ethanol (90%) 5.7 ml, H_2O 3 ml) and implanted in the right jugular vein with a catheter, consisting of

Medical-Grade tubing (Silastic, Dow Corning Corporation, Michigan, USA) according to the technique previously described (Lecca et al., 2006). A stable fixation in the mid-scapular region of the back was embedded by a polypropylene mesh (Evolution, BULEV, weight 48g/mq, Dipromed, Italy). During the recovery period, at least seven days after surgery, the catheters were daily flushed with 0.1 ml of gentamicin (40 mg/ml) and with heparinized saline (heparin 250 U/ml in 0.9% sterile saline). Ten days after recovery from surgery 14 rats were trained to self-administer JWH-018 (10µg/kg/inf, 12 ul, iv) in 1 hour-daily sessions (5 days/week) for nine consecutive sessions, according to a fixed ratio 1 schedule of reinforcement (FR 1, 1 nose-poke: 1 injection). On the 10^{th} session the self-administered dose increased to 20µg/kg/inf and at the third week, when all rats had fulfilled the criterion of 85% responses in the active hole and stable responding over three sessions, the schedule of reinforcement was increased to FR 3 (3:1) (19^{th} to 27^{th} session). Nose-poke in the active hole resulted in a 1-sec infusion of JWH-018. Each JWH-018 infusion was followed by a 20-sec *time-out* period, during which further nose-pokes were recorded but did not result in additional intravenous infusions. To investigate the involvement of CB1 cannabinoid receptors, the CB1 receptor antagonist Rimonabant (SR-141716A), was administered 30 min before putting the rat in the operant boxes for two consecutive sessions (28^{th} and 29^{th}). After these two sessions the FR 3 schedule continued as before till the 39^{th} session where the rats were studied in extinction phase. At this phase, JWH-018 solution was substituted by vehicle (39^{th} – 47^{th} sessions). A group of 6 rats were also studied in reacquisition when vehicle was replaced with JWH-018 (48^{th} – 54^{th} session). At the end of each SA session, the catheters were flushed with 0.1 ml of heparinized saline and the rats were returned to their home cages where a daily ration

of 20 g of food was made available, which maintained body weights at stable levels throughout these studies. The weight of rats at the beginning of SA studies was 300–325 g. Rats were weighed every day for the duration of the SA experiments. No significant reduction of body weight was observed.The responses performed by each rat on both holes for the entire 1-h daily session and the corresponding number of reinforcers received was recorded (Graphic State 2 software, Coulbourn instruments, PA, USA).

Mice

Daily SA sessions were carried out in chambers housed in sound proof boxes (model ENV-300; Med Associates, St. Albans, VT, USA). Chambers were equipped with two levers, and the responses on one lever (active lever) delivered one reinforcer, whereas responses on the second lever had no programmed consequences (inactive lever). A white light was placed over the active lever and a red light over the inactive one as discriminative stimuli. Prior to each daily session, the jugular catheter was flushed with 0.1 ml of sterile saline and the rats were placed in the SA box.

Mice were anaesthetized with isoflurane and a chronic intravenous catheter for mice, prepared as previously described (David et al., 2001), and implanted with an indwelling intravenous catheter in the right jugular, as described previously (Rocha et al., 1998). Three days after surgery, mice started JWH-018 self-administration under a FR1 schedule (15 or 30 ug /kg^{-1}/0.025 ml^{-1}); each daily session lasted 2 hr. After 15 consecutive days, mice were switched to the progressive ratio (PR) schedule, under which the number of active lever presses required to obtain each subsequent injection was based on the adapted exponential sequence: 3, 5, 7, 9, 12, 15, 18… (Rocha et al., 1998). PR sessions lasted for 3 hr or until mice did not complete the ratio for delivery of

one injection within 1 hr. After one session, saline was substituted for JWH-018 to test extinction of lever pressing.

Data Analysis

All the numerical data are given as mean ± SEM. Data were analyzed by utilizing repeated measures ANOVA or T-test. Results from treatments showing significant overall changes were subjected to *post hoc* Tukey tests (*in vivo* microdialysis), to Bonferroni's or Dunnett's tests (electrophysiological studies and cannabinoid-like screening effects), with significance for $p < 0.05$.

For the self administration studies nose-pokes/lever-presses emitted during each session through acquisition and extinction phases were analyzed by two-way ANOVA with nose-pokes/lever-presses (i.e., active *vs.* inactive) and *days* as within factors. Reacquisition was analyzed by two-way ANOVA, with *nose-pokes* nose-pokes/lever-presses (i.e., active *vs.* inactive) and *days* in respect to the corresponding final JWH-018 session as factors. Results from treatments showing significant overall changes were subjected to *post hoc* LSD.

Figure legends

Fig.1 (Supplemental)

Daily intake of JWH-018 (μg/kg) per session during all SA phases of JWH-018 (left Y-axis) or vehicle (μl; right Y-axis) and the correspondent number of reinforcements obtained in each 1-h session, under a FR1 or FR3 schedule, from rats that acquired JWH-018 SA. Data are expressed as mean ± SEM of μg/kg/session of JWH-018 (10 or 20 μg/kg during acquisition, after SR administration and during (reacquisition phases) or vehicle (extinction phase) intake.

Fig.2 (Supplemental)

JWH-018 self-administration by C57BL/6 mice under fixed (FR1) and progressive (PR) reinforcement schedules. (A) Number of active lever-presses during JWH-018 self-administration at FR1 (15 or 30 μg/kg/inf) during acquisition, extinction, and reacquisition phases. Bars represent the mean±SEM of the last 3 sessions at 15 or 30 μg/kg/inf or Vehicle . *$p<0.05$ vs all groups. ANOVA followed by LSD *post hoc* test. (B) Number of active (*black bars*) and inactive lever-presses (*grey bars*) during JWH-018 self-administration at FR (15 or 30 μg/kg/inf) and PR (30 μg/kg/inf) schedule of reinforcement. Bars represent the mean±SEM of the last 3 sessions at FR1 and PR. *$p<0.05$ vs inactive JWH-30; $^{\#}p<0.05$ vs all groups. ANOVA followed by LSD *post hoc* test.

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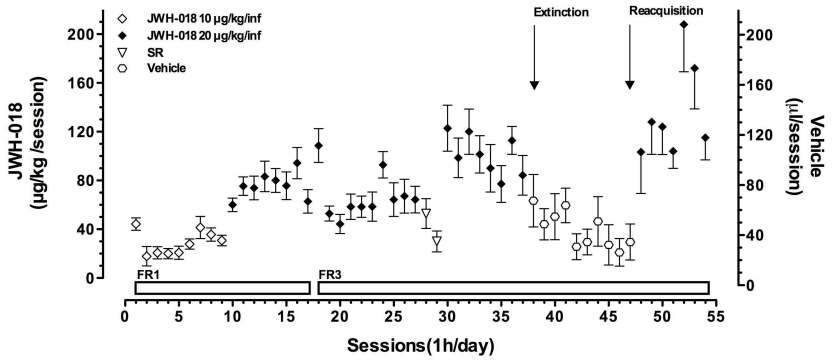

Figure 1, supplemental

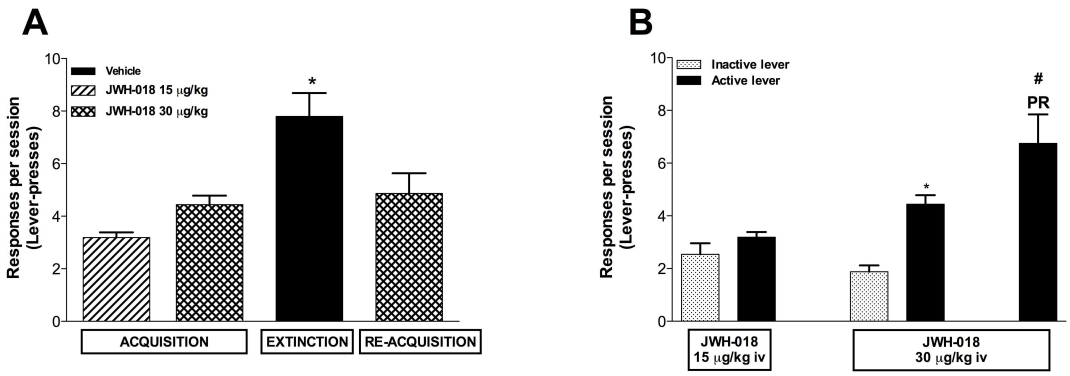

Figure 2, supplemental