Synthetic cannabinoid JWH-018 and its halogenated derivatives JWH-018-Cl and JWH-018-Br impair Novel Object Recognition in mice: Behavioral, electrophysiological and neurochemical evidence

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Abstract

It is well known that an impairment of learning and memory function is one of the major physiological effects caused by natural or synthetic cannabinoid consumption in rodents, nonhuman primates and in humans. JWH-018 and its halogenated derivatives (JWH-018-Cl and JWH-018-Br) are synthetic CB_1/CB_2 cannabinoid agonists, illegally marketed as “Spice” and “herbal blend” for their Cannabis-like psychoactive effects. In the present study the effects of acute exposure to JWH-018, JWH-018-Cl, JWH-018-Br (JWH-018-R compounds) and Δ^9-THC (for comparison) on Novel Object Recognition test (NOR) has been investigated in mice. Moreover, to better characterize the effects of JWH-018-R compounds on memory function, *in vitro* electrophysiological and neurochemical studies in hippocampal preparations have been performed. JWH-018, JWH-018-Cl and JWH-018-Br dose-dependently impaired both short- and long-memory retention in mice (respectively 2 and 24 h after training session). Their effects resulted more potent respect to that evoked by Δ^9-THC. Moreover, *in vitro* studies showed as JWH-018-R compounds negatively affected electrically evoked synaptic transmission, LTP and aminoacid (glutamate and GABA) release in hippocampal slices. Behavioral, electrophysiological and neurochemical effects were fully prevented by CB_1 receptor antagonist AM251 pretreatment, suggesting a

CB_1 receptor involvement. These data support the hypothesis that synthetic JWH-018-R compounds, as Δ^9-THC, impair cognitive function in mice by interfering with hippocampal synaptic transmission and memory mechanisms. This data outline the danger that the use and/or abuse of these synthetic cannabinoids may represent for the cognitive process in human consumer.

Keywords: JWH-018; Novel object recognition; Hippocampus; LTP; GABA/glutamate release

Abbreviations: AM251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide; CB_1R, Cannabinoid receptor 1; JWH-018, 1-pentyl-3-(1-naphthoyl)indole; JWH-018-Cl, (1-(5-chloro-pentyl)-3-(1-naphthoyl)indole); JWH-018-Br, (1-(5-bromo-pentyl)-3-(1-naphthoyl)indole); JWH-018-R, JWH-018, JWH-018-Cl and JWH-018-Br; Δ^9-THC, (−)-Δ^9-THC or Dronabinol®

Chemical compound studied in this article: JWH-018 (PubChem CID: 10382701); JWH-018-Cl (PubChem CID: 91713116); JWH-018-Br (PubChem CID: 91740913); Δ^9-THC (PubChem CID: 16078); AM251 (PubChem CID: 2125)

1 Introduction

JWH-018 (1-pentyl-3-(1-naphthoyl) indole) is a synthetic cannabinoid receptor agonist developed in the early 1990's (Huffman et al., 1994) from a computational melding of the chemical structural features of Δ^9-tetrahydrocannabinol (Δ^9-THC) with the prototypic aminoalkylindole WIN 55,212-2 (D'Ambra et al., 1992; Eissenstat et al., 1995). This aminoalkylindole is the first synthetic cannabinoid ever reported through the Early Warning System (EMCDDA, 2009; Uchiyama et al., 2010) and marketed in “Spice” and “herbal blend” for its psychoactive effects similar to those produced by Cannabis. In addition to JWH-018, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) reported to the Italian Drugs Early Warning System (NEWS) the seizure of plant material containing halogenated derivatives (N-(5-chloro-pentyl)- and N-(5-bromide-pentyl)) of the JWH-018 (EMCDDA-Europol, 2012).

JWH-018, JWH-018-Cl and JWH-018-Br (overall as JWH-018-R) bind and activate in the low nanomolar range the human CB_1 and CB_2 cannabinoid receptors (Huffman et al., 1994; Wiley et al., 1998; Vigolo et al., 2015) causing important psychiatric and physical adverse effects in consumers. Specifically, psychiatric effects are characterized by anxiety, psychosis, hallucination and alterations in cognitive abilities, while physical effects ranging as severity, from nausea to sympathomimetic-like symptoms as psychomotor agitation, diaphoresis, palpitations, tachycardia, tachyarrhythmia (Zimmermann et al., 2009; Castellanos et al., 2011; Every-Palmer, 2011; Schneir and Baumbacher, 2012; Hermanns-Clausen et al., 2013; Gurney et al., 2014; Zawilska and Wojcieszak, 2014; Tait et al., 2016), up to hyperreflexia and generalized convulsions (de Havenon et al., 2011; Simmons et al., 2011; Schneir and Baumbacher, 2012; Pant et al., 2012). *In vivo* animal studies revealed that JWH-018-R compounds reproduce the typical “tetrad” effects of Δ^9-THC as hypothermia, analgesia, hypolocomotion and akinesia (Wiley et al., 2012; Wiebelhaus et al., 2012; Brents et al., 2012; Macri et al., 2013; Vigolo et al., 2015) and impair sensorimotor responses in mice (Marti et al., 2013a; Ossato et al., 2015). JWH-018 *per se* produces anxiolysis, depressive-like behaviour (Macri et al., 2013), aggressive response (Ossato et al., 2016) and stimulates dopamine (DA) release in the nucleus accumbens (NAc) shell of mice (De Luca et al., 2015a; Miliano et al., 2016). Moreover, a preliminary study showed that JWH-018, more potently than Δ^9-THC, impaired working memory in adult mice (Marti et al., 2013b). The working memory is thought to be a short-term form of memory that develops from a short-term acquisition of trial-unique information [as in the case of Novel Object Recognition (NOR) test] which plays a crucial role in the processes of learning and memory (Baddeley, 1981; Cowan, 2008). This observation is particularly relevant since cannabinoids, such as Δ^9-THC, endocannabinoids or CB_1 receptor synthetic agonists impair learning and memory in humans (Croft et al., 2001), in nonhuman primates (Evans and Wenger, 1992) and in rodents (Fehr et al., 1976; Stiglick and Kalant, 1983; Stiglick et al., 1984; Lichtman et al., 1995; Brodkin and Moerschbaecher, 1997; Jentsch et al., 1997; Stella et al., 1997; Hampson and Deadwyler, 1998; Mallet and Beninger, 1998; Nava et al., 2000; Ciccocioppo et al., 2002; Basavarajappa and Subbanna, 2014). These detrimental effects on memory functions are thought to be associated with activation of cannabinoid receptors in the hippocampus (Hampson and Deadwyler, 1999; Egashira et al., 2002; Deadwyler et al., 2007; Wise et al., 2009), where CB_1 receptors are highly expressed (Berrendero et al., 1999). Indeed, both systemic (Heyser et al., 1993; Ferrari et al., 1999; Hampson and Deadwyler, 2000; Varvel et al., 2001) and intra-hippocampal (Lichtman et al., 1995; Egashira et al., 2002; Hampson et al., 2011) administration of cannabinoid receptor agonists induce deficits in several hippocampal memory tasks. These cognitive deficits are attributable to the negative action that exogenous cannabinoids exert on the two major kinds of hippocampal-based synaptic plasticity mechanisms such as long-term synaptic potentiation LTP (Nowicky et al., 1987; Collins et al., 1994; Terranova et al., 1995; Izumi and Zorumski, 2016) and long-term synaptic depression LTD (Misner and Sullivan, 1999; Han et al., 2012).

The present study was firstly aimed at investigating in mice the effects of acute exposure to JWH-018, JWH-018-Cl and JWH-018-Br in a working memory task, the NOR test. Moreover, in view of the obtained results and to possibly identify their neuronal and neurochemical substrates, electrophysiological and release experiments have been combined to possibly evaluate the effects of JWH-018-R compounds on: *i*) synaptic transmission in CA1 hippocampal area of mouse brain slice; *ii*) paired pulse stimulation and fiber volley in CA1 hippocampal area; *iii*) synaptic plasticity in CA1 hippocampal area of mouse brain; *iv*) GABA and glutamate release from hippocampal slices.

2 Material and methods

2.1 Animals

Male ICR (CD-1®) mice, 25–30 g (Harlan Italy; S. Pietro al Natisone, Italy), were group-housed (8–10 mice per cage; floor area per animal was 80 cm^2; minimum enclosure height was 12 cm) on a 12:12-h light-dark cycle (light period from 6:30 a.m. to 6:30 p.m.), temperature of 20–22 °C, humidity of 45–55% and were provided with ad libitum access to food (Diet 4RF25 GLP; Mucedola, Settimo Milanese, Milan, Italy) and water. The experimental protocols performed in the present study were in accordance with the U.K. Animals

(Scientific Procedures) Act, 1986 and associated guidelines and the new European Communities Council Directive of September 2010 (2010/63/EU) a revision of the Directive 86/609/EEC. Moreover experimental protocols were approved by the Italian Ministry of Health and by the Ethical Committee of the University of Ferrara. Adequate measures were taken to minimize the number of animals used, their pain and discomfort. 4–6 weeks old male mice, same strain, were used for electrophysiological and release experiments.

2.2 Drug preparation and dose selection

JWH-018 and (−)-Δ^9-THC (Dronabinol®) were purchased from LGC Standards (LGC Standards S.r.L., Sesto San Giovanni, Milan, Italy) while the CB_1 receptor antagonist AM251 was purchased from Tocris (Tocris, Bristol, United Kingdom). As previously reported (Vigolo et al., 2015), JWH-018-Cl and JWH-018-Br were both purchased on Internet (www.chemicalservices.net) in the form of powders sold with purity greater than 99%. Then the two halogenated synthetic cannabinoids were isolated and purified by chromatography (in the laboratory of Dr. C. Trapella) with a medium pressure system ISOLERA ONE (Biotage Sweden) and subsequently characterized by Agilent 6520 nano HPLC ESI-Q-TOF (Agilent Technologies) and Varian 400 MHz NMR (for the methodology see Ossato et al., 2016). Drugs for *in vivo* test were initially dissolved in absolute ethanol (final concentration = 2%) and Tween 80 (2%) and brought to the final volume with saline (0.9% NaCl). The solution made with ethanol, Tween 80 and saline was also used as vehicle. AM251 (1 mg/kg) was administered 20 min before JWH-018-R compounds and Δ^9-THC injections. Drugs were intraperitoneally (i.p.) administered at a volume of 4 µl/gr. For *in vitro* electrophysiology and release experiments, the substances were dissolved in absolute ethanol (EtOH; maximum concentration = 0.1% v/v). The used dose range of JWH-018-R compounds (0.01–1 mg/kg) or Δ^9-THC (0.1–3 mg/kg) was chosen on the basis of previous studies (Vigolo et al., 2015; Ossato et al., 2015).

2.3 Behavioral studies

2.3.1 Novel Object Recognition test

The Novel Object Recognition (NOR) test was chosen as it represents a ‘‘pure’’ working memory task, which does not involve the retention of a rule, but it is entirely based on the spontaneous exploratory behaviour of rodents towards objects (Ennaceur and Delacour, 1988; Ennaceur and Meliani, 1992; Scali et al., 1997; Ennaceur et al., 1997).

This test was performed according to the method reported by Ennaceur and Delacour (1988). The test was conducted in three phases: habituation, familiarization and choice. Firstly, CD-1 mice (n = 10/group) were subjected to a 3-day habituation phase, conducted by placing each animal into the NOR chamber (a square open field 60 cm × 60 cm × 40 cm, dark PVC plastic box) located in a dimly lit (50 lux), sound-attenuated and acclimatized room. Mice were allowed to explore freely for 20 min/day. No objects were placed in the box during the habituation trial. Twenty-four hours (hr) after last habituation section, the familiarization trial was conducted by placing the mouse in the field in which two identical objects (A, A) were positioned on the corners of the arena approximately 6 cm from the walls. Mice were placed at the mid-point of the wall opposite to the objects and allowed to explore them for 15 min. After 15 min from the familiarization phase, mice were injected with vehicle or drugs (JWH-018, JWH-018-Cl, JWH-018-Br or Δ^9-THC) and tested in two consecutive choice sections performed 2 h (short-term memory) and 24 h (long-term memory) after the drug administration. During the choice test at 2 h, one of the two familiar objects (A) was replaced with a new one (novel; B), different in shape, dimension and color. Each mouse was then placed in the apparatus and left free to explore the objects (A and B) for 5 min. In the choice test given at 24 h, the mice explored the open field for 5 min in the presence of one familiar (A) and one novel object (C, different from B). Exploration was defined as the time (sec) during which the mouse nose was in contact with the object or directed toward it at a distance ≤ 2 cm. Turning around the object was not considered as exploratory behavior.

All experiments were performed using the ANY-maze video tracking system (Ugo Basile, application version 4.99 g Beta) and subsequently analyzed by an observer blind to the mouse treatment and to which object was the novel one. Exploration time of familiar (A) and novel (B) object was detected. The novel object preference was quantified as Recognition Index (RI) calculated as: (novel B - familiar A)/(novel B + familiar A). Using this metric, scores approaching zero reflects no preference (impairment of recognition memory), positive values reflect preference for the novel object (good recognition memory) while negative numbers reflect preference for the familiar (impairment of recognition memory). Moreover, the total exploration time (sec) spent by the animal in the choice phase at 2 h (familiar A + novel B) and 24 h (familiar A + novel C) was calculated to investigate the effect of drugs on object exploration.

The objects to be discriminated by mice were 7 sets of novel and familiar objects of different material (plastic, glass or ceramic), shape (cube, parallelepiped and cylinder), dimension (height: 3–8 cm; width: 6–8) and color (light yellow, red and blue). To avoid that the material of objects could interfere with mouse's preference, we randomly used objects of different material (plastic, glass or ceramic) and we balanced the use of plastic, glass or ceramic objects among the different groups (doses and drugs). The set of objects used in the familiarization phase (two identical A, A objects) was used in the subsequent vehicle/drug conditions at 2 and 24 h. The choice of object for novel or familiar was counterbalanced and the position of each object was also alternated between trials to avoid any misinterpretation of data. The object weight was such that they could not be displaced by mice. To avoid mice olfactory cues, objects and apparatus were carefully cleaned with a dilute (5%) ethanol solution and water between animal trials and also between familiarization and choice phase (executed 2 and 24 h after the familiarization phase). Animals that spent less than 10 s exploring both objects were excluded from the study and replaced by other animals.

Since the administration of JWH-018-R compounds may blur pure mnemonic tasks in different ways [i.e. by impairing motor performance (Vigolo et al., 2015; Ossato et al., 2015), by reproducing amotivational syndrome (Miyamoto et al., 1995) and inducing anhedonia (Macri et al., 2013) in rodents, by inducing behavioral effects that overall interfere with the spontaneous exploration of a new object in the tests], the effects of JWH-018-R compounds on the spontaneous Locomotor Activity (LA) of mice during the NOR test and their ability to induce an amotivational syndrome by using the Tail Suspension (TS) test (Nowicky et al., 1987), have been also investigated.

2.3.2 Locomotor Activity

The Locomotor Activity (LA) of mice during the NOR test both at 2 h and 24 h was measured by using the ANY-maze video tracking system (ANY-maze 4.99 g Beta, Ugo Basile, Milan, Italy). The parameters measured were: total distance travelled (m), average

speed (m/sec) and total time of immobility. The animal is considered immobile when 95% of its image remains in the same place for at least 2 s.

2.3.3 Tail Suspension test

The Tail Suspension (TS) test was performed according to the method reported by (Steru et al., 1985). The posture of immobility in the mouse was originally coined 'behavioural despair' (Porsolt et al., 1977), largely based on the assumption that the animals have 'given up hope of escaping'. In the present study, to reproduce the behavioural condition present in the NOR test, mice underling TS test were previously trained on habituation (3 days) and familiarization phases. Briefly, CD-1 mice (n = 10/group), both acoustically and visually isolated, were suspended 50 cm above the floor by an adhesive tape placed approximately 1 cm from the tip of the tail. Immobility time was recorded during a 6-min period (Carbajal et al., 2009; Gehlert et al., 2009). The mice were considered immobile only when they hung passively and were completely motionless. Cannabinoid receptor agonists were injected 30 min after the familiarization phase and the TS test was executed, as for the NOR test, at 2 h and 24 h. For TS test evaluation all experiments were videotaped and the ANY-maze video tracking system was used (Ugo Basile, application version 4.99 g Beta) and scored by an observer blind to the treatment.

2.3.4 Electrophysiological studies in hippocampal slices

2.3.4.1 Tissue preparation The hippocampal transverse slice model was used to evaluate the acute effects of JWH-018-R on synaptic excitatory transmission and plasticity. Mice were deeply anesthetized with isoflurane and guillotine beheaded. After removal of the brain, hippocam were rapidly isolated and placed in ice-cold artificial cerebrospinal fluid (aCSF), of the following composition (in mM): NaCl, 126; KCl, 2; KH_2PO_4, 1.25; $NaHCO_3$, 26; $MgSO_4$, 2.0; $CaCl_2$, 2.5; D-glucose 10. All solutions were saturated with a 95% O_2/5% CO_2 gas mixture. Transversal hippocamp slices of 425 µm nominal thickness were cut with a McIlwain tissue chopper (Gomshall, U.K.). After discarding the first 2 slices obtained from dorsal hippocampal pole, the four to five following slices cut were positioned in a multi-well Haas incubation (400 ml volume) chamber at 28 °C for recove under constant O_2/CO_2 bubbling for at least 90 min until recording. To each single hippocampal slice CA1 from CA3 areas were disconnected by a surgical cut. A single slice was then transferred on a nylon mesh sited into a submerged-type recording chamber (3 ml total volume) and continuous superfused (3.0 ml/min) with warmed (32–33C°) aCSF O_2/CO_2 pre-saturated. WINLTP 2.10 computer software ~~(Anderson, 2007)~~ was used for stimulus triggering, PC recording (PCIe-6321, National Instruments, Austin TX, USA, 20 kHz sampling rate) and on/off-line potential analysis. Synapti responses of CA1 pyramidal neurons were elicited by electrical stimulation of the Schaffer collateral/commissural pathway. Pairs of stimulation pulses (80 µs duration; 0.05 Hz, 50 ms interpulse), were delivered by a DS2 constant voltage stimulus isolation unit (Digitimer, U.K.) by mean of concentric bipolar electrode (o.d. 125 µm, FHC, USA). Evoked potentials were recorded with borosilicate glass electrodes produced with a vertical puller (Kopf 750, Tujunga USA) and filled with aCSF (1.5 ± 0.5 MΩ), placed in the distal third of the stratum radiatum to record fEPSP. Distanc between stimulating and recording electrodes was 200–300 µm. Depth of the recording electrode was carefully adjusted to achieve the maximal fEPSPs response. Recorded potentials were amplified (Axoclamp2A DC-coupled - Cyberamp 320, Molecular Devices, Sunnyvale CA, USA) and filtere (5.0 kHz) prior to A/D conversion. Once obtained a stable synaptical response for at least 20 min, a stimulus\response curve (SRC) was generated as previously described (Zucchini et al., 2008) to extrapolate the stimulation intensity evoking a fEPSP 30–40% of the maximal achievable amplitud than held constant throughout the experiment. Drugs were added to reservoir and applied via bath perfusion. To investigate whether the vehicle had any effect on synaptic activity, the superfusion inlet was switched to a reservoir containing aCSF plus the amount of vehicle present for th corresponding drug concentration (sham application), before switching to the solution including also the drug, for comparison.

To evaluate modifications of synaptic plasticity we induced LTP using the theta-burst (TB5) stimulation paradigm (1 train with 5 bursts of 5 stimuli each burst, 100 Hz intraburst frequency, 5 Hz interburst (Morini et al., 2011). This protocol has the peculiarity to induce an LTP of intermediate magnitude, thus allowing detection of modulatory effects in both inhibitory and facilitatory directions and amplify CB_1-agonist effect, differently from classical high frequency stimulating protocol (FST) which hinders the memory impairment related to CB_1Rs activation (Slanina et al., 2005). Stimulation protocol was applied on two-pathways to isolate induced LTP from other drug induced pharmacological effects (Morini et al., 2011). In details, two independent synaptic inputs to the same population of CA1 pyramidal cells were activated by two stimulating electrodes positioned on opposite sites relative to the recording electrode. Input pathways were alternately stimulated every 20 s at the stimulus intensity previously identified by stimulus\response curve. After 15 min of stable baseline responses, stability of the maximal response was checked with a single pulse of maximal stimulation and baseline responses were recorded for further 10 min before the drug, antagonist or sham solution application. At 10 min of stable responses, LTP was induced through the test pathway by TB5. fEPSP was then recorded for 45 min, whereas the other, control pathway received only the test pulse at 0.05 Hz. At the end of the experiment (45 min after TB5), test and control pathways were simultaneously activated with TB10 stimulation (3 trains of 10 bursts with 5 stimuli each burst, 100 Hz intraburst frequency, 5 Hz interburst frequency, 0.05 Hz intertrain period), to evoke the maximally achievable potentiation as a control for slices viability. The response was followed for 15 min and the last 2 min were used for measuring the maximal potentiation achievable. The magnitude of maximal potentiation obtained with TB10 stimulation was also used in additional analyses to calculate TB5 stimulation-induced LTP as a fraction of maximally inducible potentiation in each slice, thereby minimizing variability due to differences in LTP susceptibility between preparations.

2.3.5 Neurochemical studies in hippocampal slices

2.3.5.1 Tissue preparation On the day of the release experiment, the animals were sacrificed by decapitation, their brain promptly isolated and 400 µm thick slices (~10 mg each) were obtained from both the left and right hippocampi, by using a McIlwain tissue chopper (Gomsha U.K.). The tissue was then allowed to equilibrate for 20 min at room temperature in Krebs' solution (composition in mM: NaCl 118; KC1 4.4; $CaC1_2$ 1.2; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25; glucose 10) and gassed with a mixture of 95% O_2 plus 5% CO_2.

2.3.5.2 Spontaneous glutamate and GABA release For the experiment on spontaneous glutamate and GABA release, the slices were transferred into oxygenated superfusion chambers (0.6 ml volume each; two-three slices/chamber, temperature 37 °C) and continuous superfused at a flow rate of 0.3 ml/min with an oxygenated Krebs' solution. After 30 min of superfusion, the experiment started by collecting superfused 5 min samples from each chamber for 60 min (twelve samples). The first three samples were used to assess basal glutamate and GABA release thereafter, JWH-018 (0.1 and 1 µM), JWH-018-Cl or JWH-018-Br (1 µM) or their vehicle (EtOH) were added to the superfusion medium and maintained until the end of the experiment (Ferraro et al., 2012).

2.3.5.3 K^+-evoked glutamate and GABA release For the experiment on the K^+-evoked glutamate and GABA release, 5 min samples were collected from the 30° to the 90° min from the onset of superfusion. During this period, the slices were stimulated twice by pulses (2 mi duration) of high potassium (20 mM) Krebs' solution (corrected for osmolarity by replacing KCl for NaCl), at the 45° (St_1) and 70° (St_2) min after the onset of superfusion. JWH-018 (0.1 and 1 µM), JWH-018-Cl or JWH-018-Br (1 µM) or their vehicle (EtOH) were included into the superfusion mediu 10 min before St_2 and maintained until the end of the experiment. When required, AM251 was added either alone or 10 min before the treatments (Ferraro et al., 2012).

2.3.5.4 Glutamate and GABA analysis Glutamate and GABA levels in the perfusated samples were measured by HPLC with fluorimetric detection. Briefly, 25 µl were transferred into glass microvials and placed in a temperature-controlled (4 °C) Triathlon autosampler (Spar Holland, Emmen, The Netherlands). Thirty µl of *o*-phthaldialdehyde/mercaptoethanol reagent were added to each sample, and 30 µl of the mixture were injected onto a Chromsep analytical column (3 mm inner diameter, 10 cm length; Chrompack, Middelburg, The Netherlands). The column wa eluted at a flow rate of 0.48 ml/min (Beckman125 pump; Beckman Instruments, Fullerton, CA, USA) with a mobile phase containing 0.1 M sodium acetate, 10% methanol and 2.2% tetrahydrofuran (pH 6.5). Glutamate and GABA were detected by means of a Jasco fluorescence spectrophotomete FP-2020 Plus (Jasco, Tokyo, Japan). The retention times of glutamate and GABA were ~3.5 and ~15.0 min, respectively.

2.4 Data and statistical analysis

Data are expressed in absolute values and are presented as the mean ± SEM or SDM when indicated. Unless indicated otherwise, the *in vivo* experiments were performed using equal number of animals per treatment (n = 10). Statistical analysis for *in vivo* results has been performed on absolute data by one-way or two-way repeated measure (RM) analysis of variance (ANOVA), as specified in figure captions. In case ANOVA yielded a significant F score, Bonferroni's post hoc test has been performed to determine group differences. Unpaired t-Student test was used to compare the vehicle-treated with untreated control groups and $p < 0.05$ was considered statistically significant.

The fEPSP amplitude was defined as the slope of the initial falling phase of the electrical response recorded following the afferent volley, and measured by linear regression in the region between 30 and 70% of the fEPSP. To calculate TB5 stimulation-induced synaptic potentiation in test pathway independent of other treatment effects (e.g. changes in excitability) which affect both inputs, we used the following procedure: for each experiment, the measured fEPSP slopes recorded from both inputs were normalized over the average of those recorded during the last 5 min period before substance application. The normalized values of control (non-potentiated) input were then subtracted from the corresponding values of the test (potentiated) input to obtain the net potentiation (i.e. LTP). Steady-state values of net potentiation produced by TB5 stimulation were obtained by averaging the values of the 11 consecutive responses recorded over the 5 min period between 40 and 45 min after TB5 stimulation. The maximally achievable potentiation was calculated by averaging the values of 5 responses over the 2 min period between 13 and 15 min after TB10 stimulation.

The effects of treatments on spontaneous glutamate and GABA release were calculated as percentages of the mean ± SEM of the mean of the first three samples. The percentages obtained from treated groups were compared with the corresponding ones obtained from untreated (control) slices assayed in parallel. When the effects of the drugs on K^+-evoked glutamate and GABA release were studied, the St_2/St_1 ratio for treated slices was calculated and compared with the corresponding St_2/St_1 value obtained from control slices assayed in parallel. K^+-evoked glutamate and GABA release was expressed as percent increase over the spontaneous (i.e. basal) glutamate or GABA release, as calculated by the mean of the two fractions collected prior to the depolarizing stimulus (Ferraro et al., 2012). The statistical analysis was carried out by analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons.

Statistical analyses were performed using GraphPad Prism software.

3 Results

3.1 Behavioral studies

3.1.1 Novel Object Recognition test

To investigate whether novel synthetic cannabinoid agonists JWH-018, JWH-018-Cl and JWH-018-Br affect memory retention in mice we performed the NOR test, comparing results with those induced by Δ^9-THC (Fig. 1). During the familiarization phase, no difference was seen in the time spent by mice to investigate the two objects (*data not shown*). There were no significant differences between vehicle-treated and control mice in the NOR test (2 h after vehicle injection: $t = 0.2456$, $df = 18$, $p = 0.8088$; and 24 h: $t = 0.1438$ $df = 18$, $p = 0.8873$; *data not shown*). In contrast, treatment with cannabinoids induced a significant impairment of recognition memory, as indicated by a RI value ≤ 0.

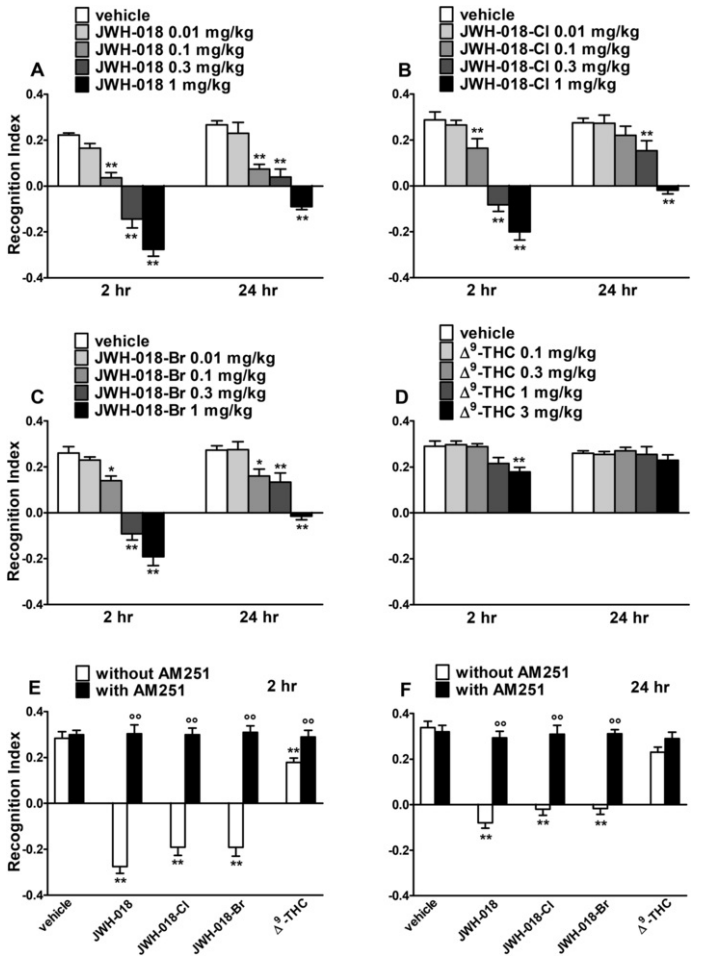

Fig. 1 Effect of systemic administration (0.01–1 mg/kg i.p.) of JWH-018 (panel A), JWH-018-Cl (panel B), JWH-018-Br (panel C) and Δ^9-THC (0.1–3 mg/kg i.p.; panel D) on Recognition Index (RI) in the NOR test in mice. JWH-018, JWH-018-Cl, JWH-018-Br and Δ^9-THC given 15 min after the familiarization phase impaired the short- (at 2 h) and long- (24 h) memory recognition in mice. AM251 (1 mg/kg i.p.) administered 20 min before agonists prevented the impairment of the RI both at 2 h (panel E) and 24 h (panel F). All drug-treated groups were compared with the respective vehicle-treated groups. Data are expressed as RI (see material and methods) and represent the mean ± SEM of 10 animals for each treatment. Statistical analysis was performed by one-way ANOVA followed by the Tukey's test for the dose response curve, while the statistical analysis of the interaction with AM251 was performed by two-way ANOVA followed by the Bonferroni's test. **p < 0.01 *p < 0.05 versus vehicle. °°p < 0.01 versus agonist administration.

NOR was impaired both at 2 and 24 h from the administration of JWH-018 ($F_{4,49}$ = 65.45; p < 0.0001 and $F_{4,49}$ = 24.53; p < 0.0001, respectively; Fig. 1-A), JWH-018-Cl ($F_{4,49}$ = 42.88; p < 0.0001 and $F_{4,49}$ = 17.16; p < 0.0001, respectively; Fig. 1-B) or JWH-018-Br ($F_{4,49}$ = 42.88; p < 0.0001 and $F_{4,49}$ = 13.78; p < 0.0001), respectively; Fig. 1-C). In particular, JWH-018 at 0.1 mg/kg significantly reduced the RI at 2 h (~16%/vs vehicle), while a reversed negative score, indicating a mouse preference toward the familiar object (A) respect to the novel one (B), was obtained following the administration of the 0.3 mg/kg (~−65%) and 1 mg/kg (~−125%; Fig. 1-A) doses. The effect of JWH-018 persisted at 24 h test, leading to a significant decrease of RI at 0.1 mg/kg (~28%) and 0.3 mg/kg (~15%), and to a RI reversion at 1 mg/kg (~−34%; Fig. 1-A). JWH-018-Cl reduced the RI at 2 h (0.1 mg/kg, ~57%) and reversed it at 0.3 (~−29%) and 1 mg/kg (~−70%; Fig. 1-B). The effect of persisted at 24 h as indicated by the RI significant reduction (0.3 mg/kg; ~56%; Fig. 1-B) or reversion (1 mg/kg; ~ −7%; Fig. 1-B). Similarly, 2 h after JWH-018-Br administration a RI reduction (0.1 mg/kg; ~54%; Fig. 1-C) or reversion (0.3 and 1 mg/kg; ~ −35% and ~ −73%, respectively; Fig. 1-C). The JWH-018-Br-induced memory impairment persisted at 24 h as indicated by the RI significant reduction (0.1 mg/kg and 0.3 mg/kg; ~59% and ~49%, respectively) or reversion (1 mg/kg; ~ −5%; Fig. 1-C). Consistently with its lower potency on CB_1 receptor in respect to JWH-018-R compounds (Vigolo et al., 2015), Δ^9-THC (0.1–1 mg/kg) was ineffective, while at a higher dose (3 mg/kg) it slightly impaired memory retention at 2 h ($F_{4,49}$ = 7.125; p = 0.0002), but not at 24 h ($F_{4,49}$ = 0.5013; p = 0.7349); Fig. 1-D).

The impairments in the NOR test performance induced by JWH-018-R compounds (1 mg/kg) and Δ^9-THC (3 mg/kg) were prevented by a pretreatment with the CB_1 receptor antagonist AM251 (1 mg/kg), both at 2 h [Fig. 1-E; significant effect of agonists

($F_{4,90}$ = 33.79, p < 0.0001), AM251 ($F_{1,90}$ = 318.8, p < 0.0001) and agonist x AM251 interaction ($F_{4,90}$ = 36.38, p < 0.0001)] and 24 h [Fig. 1-F; significant effect of agonists ($F_{4,90}$ = 23.27, p < 0.0001), AM251 ($F_{1,90}$ = 154.4, p < 0.0001) and agonist x AM251 interaction ($F_{4,90}$ = 21.83, p < 0.0001]. Also the impairments in the NOR test performance induced by JWH-018-R at 0.1 mg/kg were prevented by a pretreatment with AM251 (1 mg/kg), both at 2 h [significant effect of agonists ($F_{3,72}$ = 6.901, p = 0.0004), AM251 ($F_{1,72}$ = 47.75, p < 0.0001) and agonist x AM251 interaction ($F_{3,72}$ = 7.438, p = 0.0002)] and 24 h [significant effect of agonists ($F_{3,72}$ = 8.845, p < 0.0001), AM251 ($F_{1,72}$ = 39.39, p < 0.0001) and agonist x AM251 interaction ($F_{3,72}$ = 9.493, p < 0.0001)]. By itself, AM251 did not alter the mouse NOR test performance (*data not shown*). A dose response curve of AM251 (0.01–1 mg/kg i.p.; Fig. 1S) on the inhibition of RI induced by JWH-018 is reported in Supplementary Materials.

The Total Object Exploration (TOE) time was then calculated to investigate the effects of cannabinoid administration on the mice ability to explore the objects in the NOR test.

There were no differences in TOE time between the untreated control animals and vehicle-treated mice (2 h after the vehicle administration: t = 0.2493, df = 18, p = 0.8059; 24 h: t = 0.5098, df = 18, p = 0.6164; *data not shown*). The TOE time in the choice phase was impaired both at 2 and 24 h after the administration of JWH-018 ($F_{4,49}$ = 8.565; p < 0.0001 and $F_{4,49}$ = 9.786; p < 0.0001; Fig. 2-A), JWH-018-Cl ($F_{4,49}$ = 6.792; p = 0.0002 and $F_{4,49}$ = 8.301; p < 0.0001 Fig. 2-B) or JWH-018-Br ($F_{4,49}$ = 8.514; p < 0.0001 and $F_{4,49}$ = 8.971; p < 0.0001 Fig. 2-C). Δ^9-THC, in the same dose range, was ineffective, while at the a higher dose (3 mg/kg) slightly reduced the TOE time at 2 and 24 h ($F_{4,49}$ = 2.94; p = 0.0305 and $F_{4,49}$ = 8.745; p < 0.0001, respectively; Fig. 2-D).

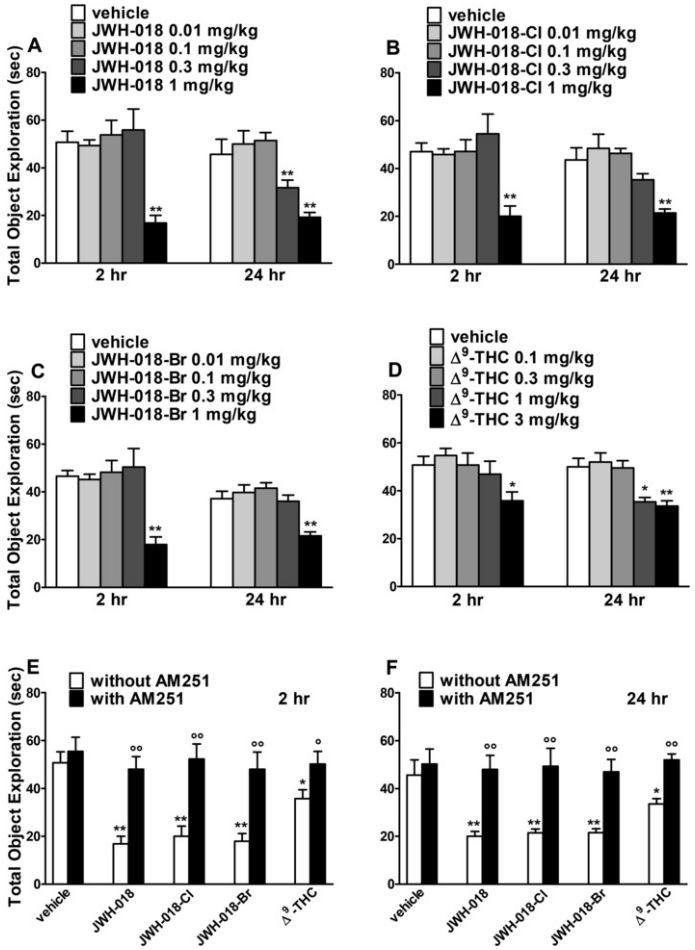

Fig. 2 Effect of systemic administration (0.01–1 mg/kg i.p.) of JWH-018 (panel A), JWH-018-Cl (panel B), JWH-018-Br (panel C) and Δ^9-THC (0.1–3 mg/kg i.p.; panel D) on Total Object Exploration (TOE) in the NOR test in mice. JWH-018, JWH-018-Cl, JWH-018-Br and Δ^9-THC given 15 min after the familiarization phase impaired the TOE both at 2 and 24 h. AM251 (1 mg/kg i.p.) administered 20 min before agonists prevented the impairment induced by cannabinoid agonists both at 2 h (panel E) and 24 h (panel F). All drug-treated groups were compared with the respective vehicle-treated groups. Data are expressed as absolute values (sec) and represent the mean ± SEM of 10 animals for each treatment. Statistical analysis was performed by one-way ANOVA followed by the Tukey's test for the dose response curve, while the statistical analysis of the interaction with AM251 was performed by two-way ANOVA followed by the Bonferroni's test. **p < 0.01, *p < 0.05 versus vehicle. °°p < 0.01, °p < 0.05 versus agonist administration.

The reductions of TOE time induced by JWH-018-R compounds (1 mg/kg) and Δ^9-THC (3 mg/kg) were prevented by the pretreatment with AM251 (1 mg/kg), both at 2 h [Fig. 2-E; significant effect of agonists ($F_{4,90}$ = 5.856, p = 0.0003), AM251 ($F_{1,90}$ = 49.6, p < 0.0001) and agonist x AM-251 interaction ($F_{4,90}$ = 2.943, p = 0.0246)] and 24 h [Fig. 1-F; significant effect of agonists ($F_{4,90}$ = 3.545, p = 0.0099), AM251 ($F_{1,90}$ = 50.08, p < 0.0001) and agonist x AM251 interaction ($F_{4,90}$ = 2.253, p = 0.0695)]. By itself, AM251 did not alter the TOE time during the NOR test (*data not shown*).

3.1.2 Locomotor activity in NOR test

The effect of JWH-018-R compounds and Δ^9-THC administration on spontaneous LA during the NOR test has been evaluated. LA was measured during the execution of the choice phase (5 min) and was performed both at 2 and 24 h after the familiarization phase. The administration of JWH-018, JWH-018-Cl, JWH-018-Br (0.01–1 mg/kg) or Δ^9-THC (0.1–3 mg/kg) affected the distance travelled (Fig. 3), the average speed (Fig. 2-S) and the immobility time (Fig. 3-S) in mice during the choice phase performed at 2 h, but not at 24 h.

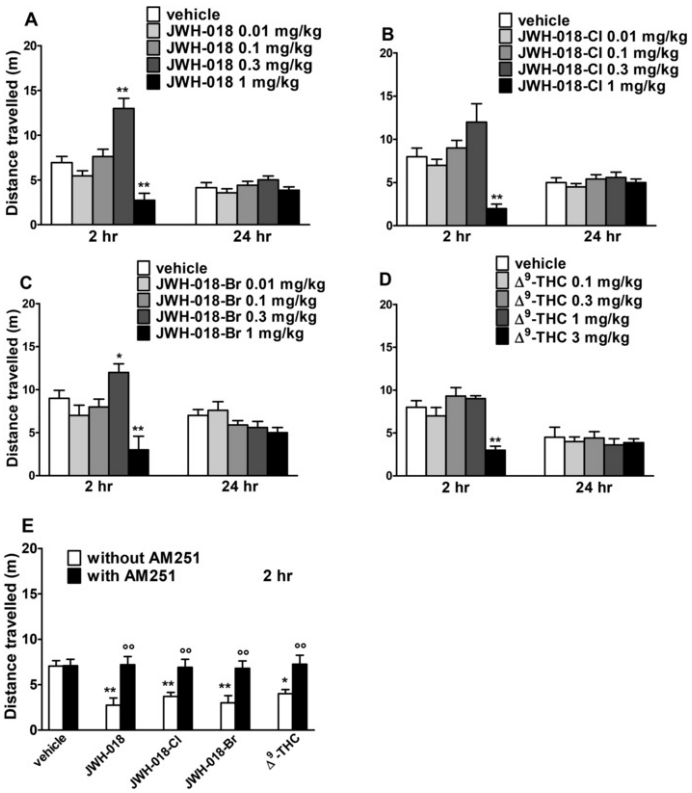

Fig. 3 Effect of systemic administration (0.01–1 mg/kg i.p.) of JWH-018 (panel A), JWH-018-Cl (panel B), JWH-018-Br (panel C) and Δ^9-THC (0.1–3 mg/kg i.p.; panel D) on the distance travelled in the NOR test in mice. JWH-018, JWH-018-Cl, JWH-018-Br and Δ^9-THC given 15 min after the familiarization phase affected the distance travelled at 2 h. AM251 (1 mg/kg i.p.) administered 20 min before agonists prevented the impairment induced by cannabinoid agonists (panel E). All drug-treated groups were compared with the respective vehicle-treated groups. Data are expressed as absolute values (m) and represent the mean ± SEM of 10 animals for each treatment. Statistical analysis was performed by one-way ANOVA followed by the Tukey's test for the dose response curve, while the statistical analysis of the interaction with AM251 was performed by two-way ANOVA followed by the Bonferroni's test. **p < 0.01, *p < 0.05 versus vehicle. °°p < 0.01, versus agonist administration.

3.1.2.1 Distance travelled Vehicle administration did not affect the spontaneous locomotion in mice in respect to untreated control animals (t = 0.1885, df = 18, p = 0.8526; *data not shown*). JWH-018 biphasically affected spontaneous locomotion in mice, increasing at 0.3 mg/kg (~187% respect to the vehicle) and reducing at 1 mg/kg (~49%) the total distance travelled by mice ($F_{4,49}$ = 20.97; p < 0.0001; Fig. 3-A). On the other hand, JWH-018-Cl administration reduced the total distance travelled by mice during the choice phase ($F_{4,49}$ = 21.93; p < 0.0001); Fig. 3-B) only at 1 mg/k (~25%). Finally, JWH-018-Br, similarly to JWH-018, biphasically affected spontaneous locomotion in mice, increasing (~133%) and reducing (~33%) at 0.3 mg/kg and 1 mg/kg, respectively, the total distance travelled by mice ($F_{4,49}$ = 8.116; p < 0.0001; Fig. 3-C). Δ^9-THC reduced the total distanc travelled by mice only at 3 mg/kg (~38%; $F_{4,49}$ = 11.33; p < 0.0001; Fig. 3-D). Treatment with AM251 (1 mg/kg) completely prevented motor changes induced by JWH-018-R compounds and Δ^9-THC [significant effect of agonists ($F_{4,90}$ = 2.744, p = 0.0332), AM251 ($F_{1,90}$ = 38.21, p < 0.0001) an

agonist x AM251 interaction ($F_{4,90}$ = 2.519, p = 0.0467); Fig. 3-E].

3.1.2.2 Average speed Vehicle administration did not affect the average speed in mice respect to untreated control animals (t = 0.6035, df = 18, p = 0.5537; *data not shown*). JWH-018 increased at 0.3 mg/kg (~166% respect to the vehicle) and reduced at 1 mg/kg (~68%) the averag speed in mice ($F_{(4,49)}$ = 10.27; p < 0.0001; Fig. 2S-A). On the other hand, JWH-018-Cl reduced (~71%) the average speed in mice ($F_{4,49}$ = 4.478; p = 0.0039; Fig. 2S-B) only at 1 mg/kg. JWH-018-Br increased (~146%) and reduced (~74%) the average speed in mice at 0.3 mg/kg and 1 mg/k respectively ($F_{4,49}$ = 6.259; p = 0.0004; Fig. 2S-C). Δ^9-THC did not affect this parameter in the NOR test (Fig. 2S-D). Treatment with AM251 (1 mg/kg) completely prevented the motor changes induced by JWH-018-R compounds [significant effect of agonists ($F_{4,90}$ = 2.735, p = 0.0337), AM25 ($F_{1,90}$ = 27.18, p < 0.0001) and agonist x AM251 interaction ($F_{4,90}$ = 2.552, p = 0.0444); Fig. 2S-E].

3.1.2.3 Immobility time Vehicle administration did not affect the immobility time in mice respect to untreated control animals (t = 0.2158, df = 18, p = 0.8315; *data not shown*). JWH-018 reduced at 0.3 mg/kg (~27% respect to the vehicle) and increased at 1 mg/kg (~247%) the immobili time in mice ($F_{4,49}$ = 8.529; p < 0.0001; Fig. 3S-A). JWH-018-Cl and JWH-018-Br similarly increased the immobility time at 1 mg/kg (JWH-018-Cl: ~188%; $F_{4,49}$ = 2.678; p = 0.0437; Fig. 3S-B; JWH-018-Br: ~202%; $F_{4,49}$ = 2.857; p = 0.0342; Fig. 3S-C). Δ^9-THC increased the immobility time in mic at 3 mg/kg (~196%; $F_{4,49}$ = 2.778; p = 0.038; Fig. 3S-D). Treatment with AM251 (1 mg/kg) completely prevented the motor changes induced by JWH-018-R compounds and Δ^9-THC [significant effect of agonists ($F_{4,90}$ = 3.665, p = 0.0082), AM251 ($F_{1,90}$ = 29.07, p < 0.0001) and agonist x AM25 interaction ($F_{4,90}$ = 2.6, p = 0.0413); Fig. 3S-E].

3.2 Tail Suspension test

The TS test was used to investigate the effect of JWH-018-R compounds and Δ^9-THC administration on mice motivation in performing a motor task. Treatment with JWH-018, JWH-018-Cl, JWH-018-Br (0.01–1 mg/kg) and Δ^9-THC (0.1–3 mg/kg) increased the immobility time in the TS test both at 2 and 24 h (Fig. 4).

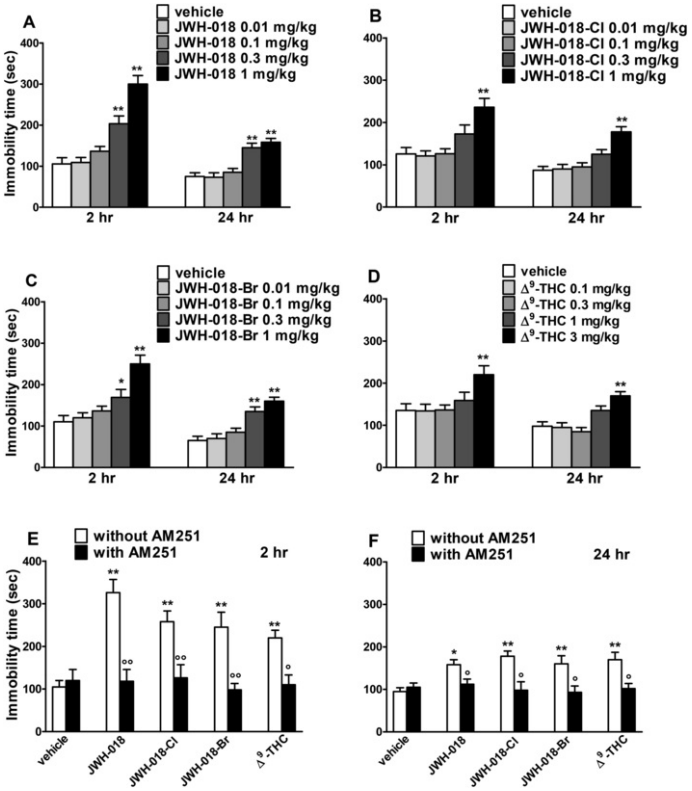

Fig. 4 Effect of systemic administration (0.01–1 mg/kg i.p.) of JWH-018 (panel A), JWH-018-Cl (panel B), JWH-018-Br (panel C) and Δ^9-THC (0.1–3 mg/kg i.p.; panel D) on the Tail Suspension (TS) test in mice. JWH-018, JWH-018-Cl, JWH-018-Br and Δ^9-THC given 15 min after the familiarization phase increased the immobility time both at 2 and 24 h. AM251 (1 mg/kg i.p.) administered 20 min before agonists prevented the impairment in the TS both at 2 (panel E) and 24 h (panel F). All drug-treated groups were compared with the respective vehicle-treated groups. Data are expressed as absolute values (sec) and represent the mean ± SEM of 10 animals for each treatment. Statistical analysis was performed by one-way ANOVA followed by the Tukey's test for the dose response curve, while the statistical analysis of the interaction with AM251 was performed by two-way ANOVA followed by the Bonferroni's test. **p < 0.01, *p < 0.05 versus vehicle. °°p < 0.01, °p < 0.05 versus agonist administration.

There were no significant differences in the immobility time between untreated control animals and vehicle-treated mice (2 h after vehicle administration: t = 0.1718, df = 18, p = 0.8655; 24 h: t = 0.1407, df = 18, p = 0.8897; *data not shown*). JWH-018 significantly increased the immobility time at 2 h ($F_{4,49}$ = 25.59; p < 0.0001) at 0.3 mg/kg (~192% respect to the vehicle) and 1 mg/kg (~284%) and these effects persisted at 24 h ($F_{4,49}$ = 16.46; p < 0.0001 Fig. 4-A). JWH-018-Cl increased the immobility time both at 2 h (~189%; $F_{4,49}$ = 8.72; p < 0.0001) and 24 h (~205%; $F_{4,49}$ = 13.09; p < 0.0001) only at 1 mg/kg (Fig. 4-B). JWH-018-Br, similarly to JWH-018, significantly increased the immobility time at 2 h ($F_{4,49}$ = 12.14; p < 0.0001) at 0.3 mg/kg (~154%) and 1 mg/kg (~227%) and these effects persisted at 24 h ($F_{4,49}$ = 16.86; p < 0.0001 Fig. 4-C). The administration of Δ^9-THC significantly increased the immobility time at 2 h ($F_{4,49}$ = 4.633; p = 0.0032) only at 3 mg/kg (~163%) and the effect persisted at 24 h (~173%; $F_{4,49}$ = 11.83; p < 0.0001; Fig. 4-D). Treatment with AM251 (1 mg/kg) completely prevented the effect of JWH-018-R compounds and Δ^9-THC on the TS test both at 2 h [significant effect of agonists ($F_{4,90}$ = 4.964, p = 0.0012), AM251 ($F_{1,90}$ = 51.83, p < 0.0001) and agonist x AM251 interaction ($F_{4,90}$ = 5.141, p = 0.0009] and 24 h [significant effect of agonists ($F_{4,90}$ = 3.093, p = 0.0196), AM251 ($F_{1,90}$ = 39.16, p < 0.0001) and agonist x AM251 interaction ($F_{4,90}$ = 3.984, p = 0.0051); Fig. 4-E].

3.3 *In vitro* studies

3.3.1 Effects of JWH-018-R compounds and Δ⁹-THC on synaptic transmission in CA1 hippocampal area of mouse brain slice

Changes in average slope amplitude of evoked CA1 synaptic population response are reported in Fig. 5. JWH-018 induced a concentration-dependent depressive effect on fEPSP (Fig. 5-B). In particular, fEPSP decline started 3–5 min after contact with JWH-018 1 μM, reaching a steady state after 40/45 min. In 4 experiments with 1 μM JWH-018, the first 2–5 min after drug contact where characterized by transitory fEPSP increase of variable amplitude (10–50% maximal increase), which steeply reverted to control values after 3–5 min (data not shown). In all test analyzed JWH-018 at 1 μM reduced significatively fEPSP (53.5 ± 17.9% vs vehicle, Fig. 5-A, B). JWH-018 at lower concentrations, proportionally reduced fEPSP with a similar temporal profile (0.01 μM = 82.5 ± 11.6% vs vehicle; 0.1 μM = 66.2 ± 16% vs vehicle). For comparison, Fig. 5-A also reported the effect of vehicle on fEPSP (101.3 ± 10.1% vs aCSF). Typical traces recorded from the same experiment, under control condition and after drug application are shown in the inset of Fig. 5-A. JWH-018-Br or JWH-018-Cl (1 μM) produced similar depressive effects as the parent compound, same concentration, although with slightly slower onset. Neither JWH-018-Cl nor JWH-018-Br induced the transient hyperexcitability observed with JWH-018. Unlike the JWH-018-R compounds, Δ^9-THC (1 μM) was ineffective on fEPSP (95 ± 7% vs vehicle; Fig. 5-B). To verify the receptor selectivity of JWH-018 (1 μM), have been conducted selectivity test by adding the CB_1 receptor antagonist AM251 (2 μM) to the perfusing solution 30 min before JWH-018 and throughout the entire experiment, following the effect for the same period required to JWH-018 alone for steady state (45 min). The AM251 by itself did not modified fEPSP (*data not shown*) while apparently blocked the depression previously observed in the presence of JWH-018 (103 ± 5.5% vs vehicle; Fig. 5-B).

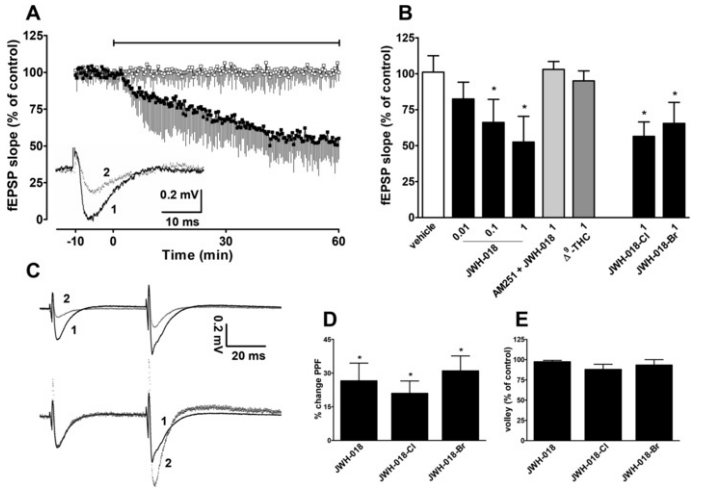

Fig. 5 Effect of JWH-018 on fEPSP of CA1 area of mouse hippocampal slice (panel A) time course effect. Each point corresponds to average value of fEPSP slope recorded at the same corresponding time, as % value respect to average value recorded before drug or vehicle application, indicated by black bar in the upper pa of the graph. Hollow dots correspond to control/vehicle conditions (n = 8), black dots corresponds to JWH-018 1 μM (n = 13). Inset shows two typical fEPSP recorded during the same experiment: control (vehicle) (5 min before JWH-018 application) = [1] - black line, 1 μM JWH-018 (45 min application) = [2] - dotted line. Histogram reporting peak values at steady state, as average fEPSP slope of last 3 min of recording (panel B). Values corresponds to % changes respect to control condition (average values of fEPSP slope 10 min before drug application). First line of X axis, is drug concentration (μM) in line two are the corresponding drugs applied. When AM251 was applied, JWH-018 1 μM was the test drug and concentration. n= (vehicle = 13, JWH-018 0.01 μM = 6, JWH-018 0.1 μM = 5, JWH-018 1 μM = 13, AM251+JWH-018 = 2, Δ^9-THC = 3, JWH-018-Cl = 3, JWH-018-Br = 4). All drug-treated groups were compared with the respective vehicle-treated group Representative superimposed traces recorded applying the paired pulse facilitation PPF paradigm (panel C). Traces are relative to the same experiment. Upper couple of traces refers to: [1] recording in control (vehicle), [2] after 45 min. JWH018 1 μM. Lower traces (SCALED) are same as upper but after normalization at first stimulus, to improve comparison of PPF effect on second stimulus response. Maximal effect on Paired Pulse Facilitation (PPF) at steady state of JWH-018, JWH-018-Cl, JWH-018-Br (1 μM; panel D). n= (JWH-018 = 5, JWH-018-Cl = 3, JWH-018-Br = 4). Values are calculated as % changes *vs* control of S2/S1 ratio (fEPSP slope of second pulse, S2 vs fEPSP of first pulse, S1). Control condition S2/S1 ratio is assumed as = 100%. Maximal effect at steady state of JWH-018, JWH-018-Cl, JWH-018-Br, on fiber volley amplitude calculated as % of amplitude before drug application (n = JWH-018 = 11, JWH-018-Cl = 3, JWH-018-Br = 4; panel E). Err

bars = s.d. values. * = p > 0.05 vs vehicle.

3.3.2 Effects of JWH-018-R compounds on paired pulse stimulation and fiber volley in CA1 hippocampal area

A paired pulse stimulation protocol was used to test for pre- or post-synaptic effects of JWH-018-R compounds. When the depressive effect of JWH-018 1 μM was at steady state, the ratio between fEPSP slope of conditioning (S1) and test pulse (S2) was modified, as shown in the example recording of Fig. 5-C (upper traces) taken from the same experiment. A statistically significant reduction of facilitatory influence of S1 over S2 may account for a pre-synaptic effect of the CB_1 receptor agonist (26.6 ± 7.8%; Fig. 5-D). A similar effect was induced by JWH-018-Cl and JWH-018-Br 1 μM (21 ± 5.5%; 31 ± 6.6%, respectively). When clearly detectable, the fiber volley amplitude has been measured, by comparing the effect of JWH-018-R compounds (1 μM) to that of vehicle (Fig. 5-E). At steady state of fEPSP, a non-significant trend to a reduction in fiber volley was observed (97 ± 1.7%; 88 ± 6.2%; 94 ± 6.6%. JWH-018, JWH-018-Cl, JWH-018-Br respectively).

3.3.3 Effect of JWH-018-R compounds on synaptic plasticity in CA1 hippocampal area of mouse brain

At steady state of the JWH-018-R effect on fEPSP, the 40% stimulation amplitude was recalculated repeating the SRC and the resulting value was then adopted for TB5 stimulation protocol to induce synaptic potentiation. Fig. 6-A shows superimposed normalized average experimental points of LTP test experiments, under control conditions and after JWH-018 (1 μM) treatment. When compared to the vehicle (85 ± 15% increase vs fEPSP slope of baseline), JWH-018 almost completely blocked the development of early and late LTP (11.3 ± 3.3%), impairing the formation of stable potentiation. Similar results were produced by JWH-018-Br and JWH-018-Cl (3.5 ± 6%; 6.5 ± 8%, respectively). In all cases, after stable fEPSP resulting from TB5 stimulation, a saturation test using TB10 protocol was applied, showing how, in the presence of JWH-018-R, it was impossible to build up any further stable potentiation, confirming the strong LTP inhibitory activity of these cannabinoids (*data not shown*).

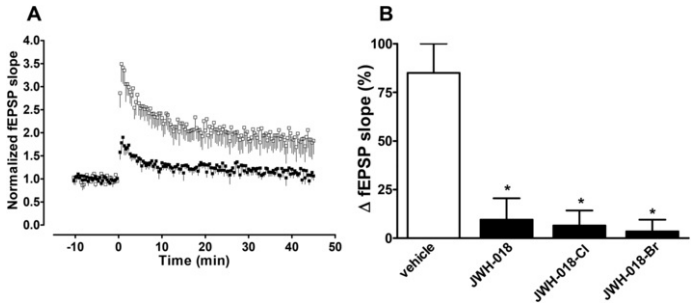

Fig. 6 Time course of fEPSP slope modification after TB5 tetanic stimulation to induce synaptic long-term potentiation. Black dots represents averaged normalized values recorded in control conditions (n = 6), hollow squares corresponds to values at steady state (45 min) after 1 μM JWH-018 (n = 13). Error bars corresponds t s.d. of corresponding averaged value (panel A). Histogram comparing averaged maximal effect on fEPSP after TB5 stimulation at steady slope, in control condition (hollow bar, n = 6) and after JWH-018 (n = 13), JWH-018-Cl (n = 3) and JWH-018-Br (n = 4), all at 1 μM (black bars; panel B). Error bars = s.d. * = p > 0.01 (vehicle vs drug).

3.3.4 Effect of JWH-018-R compounds on glutamate and GABA release in hippocampal slices

3.3.4.1 Spontaneous glutamate and GABA release In control slices, spontaneous hippocampal glutamate and GABA release slightly declined over the duration of the experiment (Fig. 7-A, B). The addition of JWH-018 (0.1 and 1 μM), JWH-018-Cl (1 μM) and JWH-018-E (1 μM) to the perfusion medium did not significantly affect spontaneous glutamate and GABA release from rat hippocampus slices (Fig. 7-A, B).

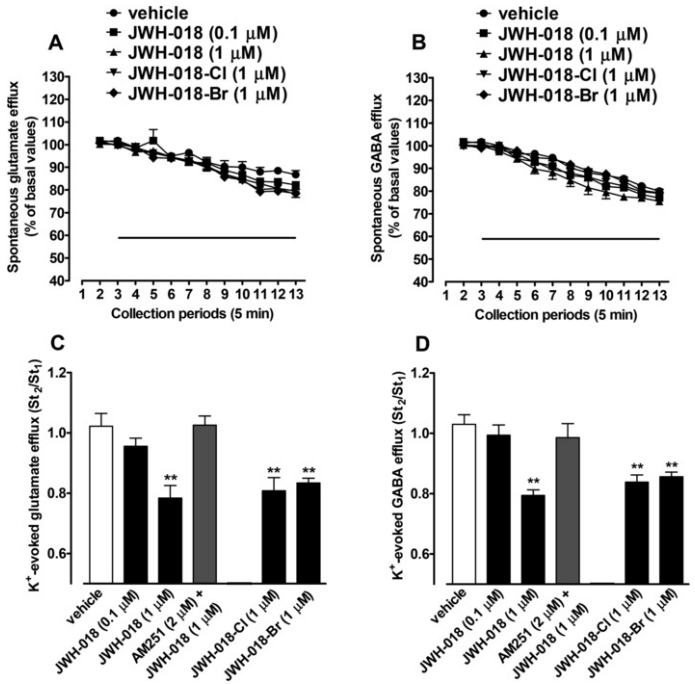

Fig. 7 Effect of JWH-018 (0.1–1 μM), JWH-018-Cl (1 μM), JWH-018-Br (1 μM) on spontaneous and K + -evoked glutamate (panel A, C) and GABA (panel B, D) release from hippocampal slices obtained from CD-1 mice. AM251 (2 μM) administered 20 min before agonists prevented the inhibitory effects of JWH-018-compounds on glutamate (panel C) and GABA (panel D) release. All drug-treated groups were compared with the respective vehicle-treated groups. Data are expressed as percentage of basal values (panel A, B) or St2/St1 Ratio (panel C, D) and represent the mean ± SEM of 5–7 animals for each treatment. **p < 0.0 significantly different from the respective vehicle group according to ANOVA followed by Newman-Keuls test for multiple comparisons.

3.3.4.2 K⁺-evoked glutamate and GABA release In control slices, a first period (2 min) of KCl (20 mM) stimulation (St_1) induced a significant increase of glutamate and GABA release (151 ± 8% and 147 ± 8% of basal values, respectively), which was quite similar to that observe during a second period of stimulation (St_2), the St_2/St_1 ratio being close to unity (1.04 ± 0.05 and 1.08 ± 0.06, respectively). When JWH-018 (1 μM) was added to the perfusion medium 10 min before St_2, a significant decrease of K^+-evoked glutamate and GABA release was observed. At a lowe concentration (0.1 μM), JWH-018 did not significantly affect K^+-evoked glutamate and GABA release (Fig. 7-C, D). To verify the receptor selectivity of JWH-018, the experiments have been repeated in the presence of AM251. As shown in Fig. 7-C and D, when AM251 (2 μM) was added to th perfusion solution 10 min before JWH-018 (1 μM), it completely blocked the effects of the agonist. By itself, AM251 (2 μM) did not affect K^+-evoked glutamate and GABA release (*data not shown*).

Finally, similarly to JWH-018, JWH-018-Br and JWH-018-Cl (1 μM) significantly decreased K^+-evoked glutamate and GABA release (Fig. 7-C, D).

4 Discussion

The present study demonstrates, for the first time, that JWH-018 or its halogenated derivatives (JWH-018-Cl and JWH-018-Br) dose-dependently and more potently than Δ^9-THC impair short- (2 h) and long-term (24 h) working memory in mice. These alterations can be directly correlated to CB_1 receptor activation since they were prevented by the selective CB_1 receptor antagonist AM251 (Gatley et al., 1996). *In vitro* studies in a hippocampal slice model confirmed that JWH-018 affects the synaptic excitatory transmission thus impairing the induced synaptic plasticity, possibly through the alteration of local neurotransmission (Hoffman et al., 2016; Kawamura et al., 2006). This is supported by the demonstration that JWH-018 reduced K^+-evoked glutamate and GABA release from hippocampal slices, a finding that to our knowledge was not previously reported in literature. Superimposable *in vitro* results have been obtained with the JWH-018 halogenated derivatives, present as well in the psychoactive Spice formulations.

The observed JWH-018-R compound-induced impairments of working memory in mice are consistent with previous studies showing the amnesic profile of natural and synthetic cannabinoid agonists in rodents (Fehr et al., 1976; Stiglick and Kalant, 1983; Stiglick et al., 1984; Heyser et al., 1993; Lichtman et al., 1995; Brodkin and Moerschbaecher, 1997; Jentsch et al., 1997; Stella et al., 1997; Mallet and Beninger, 1998; Nava et al., 2000; Ciccocioppo et al., 2002; Hampson and Deadwyler, 2000; Hampson et al., 2011; Basavarajappa and Subbanna, 2014).

JWH-018-R compounds (0.1 mg/kg) did not reduce the mouse spontaneous locomotion during the NOR test, while they impaired short- (2 h) and long-term (24 h) working memory. This finding highlights the detrimental effects of these synthetic cannabinoids on the memory functions. Moreover, the observation that the JWH-018-R compounds at the same dose (0.1 mg/kg) did not affect the immobility time of the mouse in the TS tests, showing a motor activity similar to that observed during spontaneous (Ossato et al., 2015) and stimulated motor activity (Vigolo et al., 2015), further support the negative impact of these cannabinoids on cognitive functions. Notably, and in line with this view, at the higher dose of 0.3 mg/kg, the JWH-018-R compound-induced working memory impairment was not related to an increase in TOE time, although a facilitation of locomotion activity was observed.

It is known that attention and motivation processes are likely to prioritize novelty detection in rodents (Ennaceur, 2010). Furthermore, the administration of synthetic cannabinoids decreases the motivation and causes anhedonia in mice (Macri et al., 2013). In line with this, the JWH-018-R compounds increased the immobility time of the mouse in the TS test. However, this effect is induced at a dose (0.3 mg/kg) that impaired the memory in mice without depressing motor activity and reducing the TOE time. This suggests that memory impairment caused by JWH-018-R compounds up to 0.3 mg/kg is independent from their effects on locomotion and reaction time in the TS test. On the other hand, the RI changes in the NOR test induced by JWH-018-R compounds at the dose of 1 mg/kg after 2 h, might be due to a reduction in the locomotion of mice. However, this is not the case of the cognitive impairments observed 24 h after the injection of these compounds, at the same dose.

The JWH-018-R compounds induced a biphasic profile on motor activity, characterized by a facilitation at 0.3 mg/kg and an inhibition at 1 mg/kg, fits well with the time- and dose-dependent biphasic effects that cannabinoid receptor agonists produce on movement in rodents (Rodriguez de Fonseca et al., 1998). A similar biphasic effect on movement has also been reported both for the endogenous ligand of the cannabinoid receptor anandamide (Sulcova et al., 1998), Δ^9-THC (Ossato et al., 2015) and for the synthetic compound WIN 55,212-2 (Drews et al., 2005), suggesting that this modulation is a generalized effect of cannabinoids (Rodriguez de Fonseca et al., 1998).

At the present, we cannot ruled out the possibility that other behavioral changes induced by JWH-018-R compounds may interfere with the performance of mice in the NOR test. In fact, JWH-018 (0.1 mg/kg) reduced visual, auditory and tactile sensorimotor responses in mice (Ossato et al., 2015). However, these sensorimotor changes completely disappeared 24 h after JWH-018 administration (*personal unpublished data*), while the working memory impairment induced by the compound was still detectable after 24 h of its administration. Moreover, JWH-018 (0.1 mg/kg) did not affect the TOE time. Taken together, these data suggest that the cognitive deficits observed in NOR test, at least at 24 h, are likely correlated to an inhibition of processes that are involved in memory formation and retention rather than to an impairment of motor and sensorimotor functionalities.

It is worth noting that the JWH-018-R compounds were administered at a sufficient time (15 min) to acquire memory of the objects (A, A) during the familiarization phase (Ennaceur, 2010). The evidence that at higher doses (0.3 and 1 mg/kg) JWH-018-R compounds caused a greater exploration of the familiar object compared to the new one (RI reversion) could be due to the fact that the drug-induced impairment in the already-acquired memory (Ennaceur, 2010). However, our data are not able to support this evidence, and it cannot be rule out that this effect could be related to detrimental effects induced by JWH-018-R compounds on sensorimotor functions (Ossato et al., 2015) or on their rewarding properties in rodents (De Luca et al., 2015b; Ossato et al., 2016; Miliano et al., 2016). In fact, we recently demonstrated that JWH-018 (0.3 mg/kg i.p.) stimulates dopamine transmission in the NAc shell and it served as a reinforcer in a self-administration paradigm in mice (De Luca et al., 2015a). Therefore, it is possible that the mouse associates the familiar object (A) to the rewarding drug, thus spending more time to explore that object than the novel one (B or C) in the NOR test. Further studies are necessary to clarify this aspect.

In line with the present data, the acute administration of JWH-081 (Aung et al., 2000; Huffman et al., 2005), a synthetic cannabinoid found in “Spice” and “K2” (Auwarter et al., 2009; Hermanns-Clausen et al., 2013), also impairs NOR in mice (Basavarajappa and Subbanna, 2014). In particular, JWH-081, administered 30 min before the behavioral test at 1.25 mg/kg, causes a RI reversion 1 and 4 h after its administration, being this effect no more detectable after 24 h. The longer duration of action of JWH-018-R compounds (present study) compared to JWH-081 (Basavarajappa and Subbanna, 2014) is probably due to different pharmacokinetic characteristics of these cannabinoids rather than their diverse pharmacodynamic properties, also in view of the fact that the affinity of JWH-018-R compounds for CB_1 receptor (Wiley et al., 1998; Vigolo et al., 2015) is lower than that of JWH-081 (Ki = 1.2 nM; (Aung et al., 2000; Huffman et al., 2005). In fact, it is well known that the synthetic cannabinoids of the JWH-R class are metabolized and bioactivated in the liver to monohydroxylated compounds that, as for the JWH-018 (Wintermeyer et al., 2010), *in vivo* display high affinity and agonist activity at CB_1 receptors similar to those of the parent drug (Brents et al., 2011). In contrast, other synthetic cannabinoids, such as JWH-073, are bioactivated in monohydroxylated compounds which in part maintain an agonist-like profile and in part show antagonist properties on CB_1 receptors, thus being capable of selectively shut down some biological effects of the parent drug (Brents et al., 2012). Therefore, JWH-018-R compounds may be bioactivated to agonist ligands at CB_1 receptors that could maintain the amnesic effect over the time, while JWH-081, similarly to JWH-073 (Brents et al., 2012), could generate metabolites with antagonistic activity at CB_1 receptors that could extinguish the amnesic effect of the parent drug. Another possibility is that the different duration of memory impairment induced by JWH-018-R compounds and JWH-081 could be due to the use of different mouse strain since in the current study CD-1 mice have been used to test the effects of JWH-018-R compounds, while JWH-081 was tested in C57BL/6J (Basavarajappa and Subbanna, 2014). However, in contrast to other memory tasks, the NOR test appears to be less strain-dependent and sufficiently reproducible among different mouse strains (Sik et al., 2003). This aspect is also confirmed by the fact that in the present study CD-1 mice, similarly to the C57BL/6J mice used in the NOR studies with JWH-081 (Basavarajappa and Subbanna, 2014), retain memory for the familiar object even at 24 h.

According to previous *in vivo* findings (Fantegrossi et al., 2014; Marshell et al., 2014; Vigolo et al., 2015; Ossato et al., 2015) the present study indicates that JWH-018-R compounds are more potent than Δ^9-THC in impairing working memory in rodents.

The present *in vitro* results demonstrate that JWH-018-R compounds consistently affected the synaptic excitatory transmission in a mouse hippocampal slice preparation, thus extending to halogenated derivatives recently published data on

JWH-018 (Hoffman et al., 2016). This effect mainly consisted in a significant depression of the fEPSP of superimposable extent among the different aminoalkylindoles, where a slower onset phase differentiates the halogenated derivatives. In several experiments with the highest concentration of JWH-018 tested (1 μM), has also been observed a relatively short transient increase of the fEPSP, characterized by a large variability both in amplitude and time required to extinction.

The observed selective reduction of Paired Pulse Facilitation (PPF) ratio, without a significant modification of fiber volley, suggests that JWH-018-R compounds act at presynaptic level. This also excludes a possible role of fiber volley alterations in the observed fEPSP depression. The CB_1 receptor selectivity of JWH-018 has been confirmed by the selective CB_1 antagonist AM251, which completely blocked the effects previously described. The *in vitro* experiments also show that in the same preparation, JWH-018-R (1 μM) almost completely suppressed the electrically induced LTP. Previous findings demonstrated that repeated exposure to Δ^9-THC disrupts hippocampal LTP and alters signaling at both glutamatergic and GABAergic synapses (Hoffman et al., 2007). Thus, in the present study, the effects of JWH-018-R compounds on hippocampal glutamate and GABA release have been evaluated. The results indicate that either JWH-018 or its halogenated derivatives significantly decreased K^+-evoked glutamate and GABA release. The evidence that, under the present *in vitro* experimental conditions, JWH-018-R compounds affected K^+-evoked, but not spontaneous, glutamate and GABA release, suggest that the drugs preferentially acts by interfering with the neurosecretory coupling mechanisms, rather than affecting astrocytic aminoacid efflux or glutamate and GABA leakage from nerve terminals. This results are in line with previous findings demonstrating that CB_1 receptor agonists induce, in the hippocampus, depressive effects on synaptic glutamatergic (Nowicky et al., 1987; Collins et al., 1994; Terranova et al., 1995; Stella et al., 1997; Hajos et al., 2001; Diana et al., 2003; Domenici et al., 2006; Peterfi et al., 2012) and GABAergic (Hoffman and Lupica, 2000; Chevaleyre and Castillo, 2003; Hill et al., 2007; Laaris et al., 2010; Peterfi et al., 2012) transmission. Similarly, *in vivo* experiments on hippocampus (Abush and Akirav, 2010; Jacob et al., 2012) showed significant cannabinoid-induced depressive effects on aminoacidergic signalling.

JWH-018, which has similar affinity for human and mouse CB_1 receptor (Vigolo et al., 2015), has been previously tested on different mouse slice preparations, showing a consistent inhibitory activity on excitatory synaptic transmission at different concentrations, ranging from (IC_{50}) 14.9 nM (Atwood et al., 2010) to 1.121 μM (Irie et al., 2015). This difference could be correlated to the different models used. The results reported by Irie et al. (2015), are quite close to those obtained in the present study, showing an EC_{50} values of ~1.5 μM, despite the author used cerebellar slices and the known difference in CB_1 receptor density between the hippocampal Schaffer collaterals and the climbing fibers of cerebellum. JWH-018-Cl and JWH-018-Br show similar activity as the parent compound, according to binding and behavioral results (Vigolo et al., 2015).

The maximal effect on fEPSP depression by halogenated JWH-018-R compounds is reached later in respect to JWH-018, in line with the results obtained in the present *in vivo* experiments. Interestingly, the unwanted *in vivo* toxic symptoms of JWH-018 are also produced by the halogenated compounds, but with less intensity and higher latency in respect to the parent compound. Therefore, the present data strengthen the hypothesis that halogenated derivatives may have been placed on the illegal market to try to replace JWH-018 because of its severe side effects (convulsions) that have limited its use by consumers (Ossato et al., 2015; Vigolo et al., 2015).

In the present study, Δ^9-THC (1 μM) failed to affect fEPSP. This is in contrast with the results reported by (Hoffman et al., 2016) where the same Δ^9-THC concentration exerted a clear inhibitory effect. This difference could be possibly due to the different vehicle used to solubilize Δ^9-THC in the present experiments (EtOH) and in the study by Hoffman et al. (DMSO), coupled to the partial agonist activity of Δ^9-THC at CB_1 receptors (Laaris et al., 2010). In fact, EtOH exerts an occluding effect on CB_1R (Basavarajappa et al., 2008), while DMSO is devoid of this activity. This is also supported by the evidence that under the present conditions (EtOH as vehicle), JWH-018 displayed a less depressive activity than that reported by Hoffman et al. (DMSO as vehicle). Furthermore, Hoffman et al. used an A1 receptor antagonist to block the endogenous adenosine, whose presence in the hippocampus impairs the inhibition of glutamate release mediated by CB_1R. (Hoffman et al., 2010). This could also explain the different inhibition of fEPSP produced by Δ^9-THC and JWH-018 sensitivity between their and present experiments.

In the present study has been observed in some experiments an early effect of JWH-018 (1 μM) consisting in a transient but clearly detectable increase of fEPSP, effect not displayed by halogenated JWH-018. It is likely that the activation of presynaptic CB_1R, present on GABA terminals (Pistis et al., 2002; Trettel and Levine, 2002; Chevaleyre and Castillo, 2003; Peterfi et al., 2012; Laaris et al., 2010), is on the base of this event, suggesting the future evaluation of the activity of JWH-018 on GABA terminals. This transitory fEPSP increase more clearly indicates the evidence of the prompt access of JWH-018 to the slice core, followed only later by a slower developing depression of fEPSP. This rules out the hypothesis of a slow access to whole tissue as explanation of the long time required for the steady state effect. However, JWH-018 compared to other known synthetic cannabinoids shows a peculiar activity on *in vivo* EEG parameters (Uchiyama et al., 2012), with a similar or even less potency, but characterized by a faster on/off activity. Interestingly, halogenated JWH-018-R compounds never showed this transitory hyperexcitability, possibly due to their slower onset of activity. At present, the JWH-018-induced time discrepancy between GABA and glutamate inhibition, suggested by the electrophysiological experiments, is difficult to be explained and only some suggestions can be proposed. The inhibition of GABA possibly results from the inhibitory modulation of N-type voltage-dependent calcium channels by G-proteins βg-subunits (Hoffman and Lupica, 2000; Wilson et al., 2001). Indeed, Daigle (Daigle et al., 2008) shown that by activating CB_1 receptors JWH-018 activates ERK1/2 MAPK, with a typical rapid time course (peak in 5/10 min). This rapid activation is straightforward linked to GABA currents, supporting so a direct correlation with the observed transient disinhibition. On the other hand, the depressive activity of excitatory transmission seems to undergo through a different mechanism if compared with the inhibitory one, looking the slower onset and the persistency of the excitatory depression.

The intense, rapid to emerge but transitory hyperactivity and hyperresponsivity observed *in vivo* more than *in vitro* upon CB_1 receptor activation, could be related to disinhibition seen in other different brain area like raphe, cerebellum and amygdala, where CB_1 receptors presence and activity has been demonstrated (Azad et al., 2003; Domenici et al., 2006; Irie et al., 2015).

The PPF results are in line with the expected effects of a CB_1 receptor agonist (Atwood et al., 2010, 2011), with a clear indication of a presynaptic mechanism involving the reduction of neurotransmitter release (Shen et al., 1996).

Similarly to other CB_1 agonist (Takahashi and Castillo, 2006), JWH-018-R compounds, even at 1 μM, did not significantly reduce the fiber volley amplitude. This indirectly confirm the selective effect of these compounds on presynaptic CB_1 receptors (Nemeth et al., 2008), thus possibly excluding the involvement of unspecific mechanisms.

The direct correlation between CB_1 receptor activation and hippocampal LTP impairment has been demonstrated *in vitro* and *in vivo* using different cannabinoid agonists (Nowicky et al., 1987; Collins et al., 1994; Terranova et al., 1995; Ievglevskyi et al., 2012; Navakkode and Korte, 2014), including JWH-018 and compounds of the same chemical class (Basavarajappa and Subbanna, 2014). In line with these studies, the present data shows that JWH-018-R compounds can severely affect both early- and late-LTP. Interestingly, it has been shown that CB_1 receptor activation, by inhibiting glutamate release, mainly affects the late-LTP (Misner and Sullivan, 1999). However, even a short CB_1 receptor agonist pretreatment inhibits protein synthesis via a cholinergic mechanism, resulting in a complete deletion of the late LTP (Navakkode and Korte, 2014). Thus, CB_1 receptor activation impairs glutamatergic transmission and, via NMDA receptors, mainly impairs early-LTP. Once early-LTP is blocked, also the late-LTP is of difficult induction/retention even if of differently originating mechanism. Differently from Navakkode and Korte, we applied JWH-018 for a quite long time before high-frequency stimulation-induced LTP, allowing CB1 receptor stimulation and affecting fEPSP at steady state, while they applied the agonists (WIN55,212-2) for a very short period without to evaluate fEPSP modifications. It is worth noting that JWH-018 affects LTP differently than other commonly tested synthetic cannabinoids, by affecting both early- and late-LTP. Although JWH-018 interferes with CB_1 receptors with high affinity (Aung et al., 2000; Atwood et al., 2010), the long contact time needed to reach a stable fEPSP could implicate that it enters the cell and alters LTP also through a different mechanism, possibly involving protein synthesis or activating intracellular CB_1R (Leterrier et al., 2004). Further experiments are necessary to elucidate this aspect. Robinson et al., 2007 showed straightforward correlation between CB_1 receptor activation by synthetic cannabinoid HU210 with abnormal hippocampal cell firing. These effects are also associated with induced behavioural negative effects with severe spatial memory deficits. Thus, correlation between altered aminoacidergic hippocampal transmission and cognitive function deficits has been documented since time (Hajos et al., 2001; Puighermanal et al., 2009), supporting the hypothesis of a cooperative CB_1 receptors effects on excitatory and inhibitory hippocampal network leading to LTP impairment and, as a consequence, cognitive deficits (Hoffman et al., 2016).

5 Conclusion

These behavioral, electrophysiological and neurochemical data demonstrate that synthetic JWH-018-R compounds, as Δ^9-THC, impair cognitive function in mice by interfering with hippocampal synaptic transmission and memory mechanisms.

Although obtained in animal model, these data outline the danger that the use and/or abuse of these synthetic cannabinoids may represent for the cognitive process in human consumer.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2016.06.027.

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Appendix A. Supplementary data

The following is the supplementary data related to this article:

Multimedia Component 1

Highlights

- JWH-018-Cl and -Br are new halogenated cannabinoids seized in Internet Market.
- JWH-018-R compounds impair short and long term working memory in mice.
- JWH-018 reduced K^+-evoked glutamate and GABA release from hippocampal slices.
- JWH-018-R compounds affected the synaptic excitatory transmission in mouse model.

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