**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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**Abbreviations**

| | |
|---|---|
| AM 251 | 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide |
| 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 |
| $\Delta^9$-THC | (-)-$\Delta^9$-THC or Dronabinol® |

**Keywords:** JWH-018; Novel Object Recognition; hippocampus; LTP; GABA; glutamate release

**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 assumption 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 $\Delta^9$-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 hours after training session). Their effects resulted more potent respect to that evoked by $\Delta^9$-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 AM 251 pretreatment, suggesting a $CB_1$ receptor involvement. These data support the hypothesis that synthetic JWH-018-R compounds, as $\Delta^9$-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.

## 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 $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-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 $\Delta^9$-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 $\Delta^9$-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 $\Delta^9$-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; 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 (Lichtman et al., 1995; Egashira et al., 2002; Wise et al., 2009), where $CB_1$ receptors are highly expressed (Berrendero et al., 1999). Indeed, both systemic (Iwasaki et al., 1992; Lichtman et al., 1995; Ferrari et al., 1999); Varvel et al., 2001; Da Silva and Takahashi, 2002) and intra-hippocampal (Lichtman et al., 1995; Egashira et al., 2002; Suenaga and Ichitani, 2004) 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, 25-30g (Harlan Italy; S. Pietro al Natisone, Italy), were group-housed (8 to 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 AM to 6:30 PM), 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 (-)-$\Delta^9$-THC (Dronabinol®) were purchased from LGC Standards (LGC Standards S.r.L., Sesto San Giovanni, Milan, Italy) while the $CB_1$ receptor antagonist AM 251 was purchased from Tocris (Tocris, Bristol, United Kingdom). As previously reported (Vigolo et al., 2015), JWH-018-Cl and JWH-018-Br were purchased on Internet, 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 400MHz NMR. 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. AM 251 (1 mg/kg) was administered 20 minutes before JWH-018-R compounds and $\Delta^9$-THC injections. Drugs were intraperitoneally (i.p.) administered at a volume of 4µl/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 $\Delta^9$-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; Bartolini et al., 1996).

This test was performed according to the method reported by Ennaceur and Delacour (1988) and Antunes and Biala (2011). 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 x 60 cm x 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 (hrs) 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 $\Delta^9$-THC) and tested in two consecutive choice sections performed 2 hrs (short-term memory) and 24 hrs (long-term memory) after the drug administration. During the choice test at 2 hrs, 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 hrs, 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 4.99g 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 hrs (familiar A + novel B) and 24 hrs (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). 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 hrs. 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 hrs 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 hrs and 24 hrs was measured by using the ANY-maze video tracking system (ANY-maze 4.99g 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 sec.

### 2.3.4. 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 mins after the familiarization phase and the TS test was executed, as for the NOR test, at 2 hrs and 24 hrs. For TS test evaluation all experiments were videotaped and the ANY-maze video tracking system was used (Ugo Basile, application version 4.99g Beta) and scored by an observer blind to the treatment.

### 2.3.5 Electrophysiological studies in hippocampal slices

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, hippocampi 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 hippocampal 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 recovery 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 continuously 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. Synaptic 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 a 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. Distance 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 filtered (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 amplitude, than held constant throughout the experiment. Drugs were added to reservoir and applied via bath perfusion. All parts exposed to drug before slice contact were on glass to minimize drugs capture. 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 the 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 frequency). 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 exacerbates CB1-agonist effect, differently from classical high frequency stimulating

protocol (FST) which hinders the memory impairment related to CB1Rs activation (Slanina et al., 2005). Stimulation protocol was applied on two-pathways to isolate from other pharmacological effects of drugs (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 sec at the stimulus intensity previously identified by SRC. 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 almost 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.

### 2.3.6. Neurochemical studies in hippocampal slices

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 (Gomshall, 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% $CO_2$.

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 continuously 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).

$K^+$-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 min 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 medium 10 min before $St_2$ and maintained until the end of the experiment. When required, AM 251 was added either alone or 10 min before the treatments (Ferraro et al., 2012).

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 (Spark 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 was 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 spectrophotometer FP-2020 Plus (Jasco, Tokyo, Japan). The retention times of glutamate and GABA were ~ 3.5 and ~ 15.0 min, respectively.

#### 2.3.7. 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 40-45 min after TB5 stimulation. The maximally achievable potentiation was calculated by averaging the values of 5 responses over the 2 min period between 13-15 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 $\Delta^9$-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 hrs after vehicle injection: t=0.2456, df=18, p = 0.8088; and 24 hrs: 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 $\leq 0$.

NOR was impaired both at 2 and 24 hrs 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 hrs (~ 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 hrs (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 hrs 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 hrs 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 hrs 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), $\Delta^9$-THC (0.1-1 mg/kg) was ineffective, while at a higher dose (3 mg/kg) it slightly impaired memory retention at 2 hrs ($F_{4,49}=7.125$; $P=0.0002$), but not at 24 hrs ($F_{4,49}=0.5013$; $P=0.7349$); fig. 1-D).

The impairments in the NOR test performance induced by JWH-018-R compounds (1 mg/kg) and $\Delta^9$-THC (3 mg/kg) were prevented by a pretreatment with the $CB_1$ receptor antagonist AM 251 (1 mg/kg), both at 2 hrs [fig. 1-E; significant effect of agonists ($F_{4,90}=33.79$, $p<0.0001$), AM 251 ($F_{1,90}=318.8$, $p<0.0001$) and agonist x AM 251 interaction ($F_{4,90}=36.38$, $p<0.0001$)] and 24 hrs [fig. 1-F; significant effect of agonists ($F_{4,90}=23.27$, $p<0.0001$), AM 251 ($F_{1,90}=154.4$, $p<0.0001$) and agonist x AM 251 interaction ($F_{4,90}=21.83$, $p<0.0001$]. By itself, AM 251 did not alter the mouse NOR test performance (*data not shown*).

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 hrs after the vehicle administration: $t=0.2493$, $df=18$, $P=0.8059$; 24 hrs: $t=0.5098$, $df=18$, $P=0.6164$; *data not shown*). The TOE time in the choice phase was impaired both at 2 and 24 hrs 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). $\Delta^9$-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 hrs ($F_{4,49}$= 2.94; P=0.0305 and $F_{4,49}$= 8.745; P<0.0001, respectively; fig. 2-D).

The reductions of TOE time induced by JWH-018-R compounds (1 mg/kg) and $\Delta^9$-THC (3 mg/kg) were prevented by the pretreatment with AM 251 (1 mg/kg), both at 2 hrs [fig. 2-E; significant effect of agonists ($F_{4,90}$=5.856, p=0.0003), AM 251 ($F_{1,90}$=49.6, p<0.0001) and agonist x AM-251 interaction ($F_{4,90}$=2.943, p=0.0246)] and 24 hrs [fig. 1-F; significant effect of agonists ($F_{4,90}$=3.545, p=0.0099), AM 251 ($F_{1,90}$=50.08, p<0.0001) and agonist x AM 251 interaction ($F_{4,90}$=2.253, p=0.0695)]. By itself, AM 251 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 $\Delta^9$-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 h and 24 h after the familiarization phase. The administration of JWH-018, JWH-018-Cl, JWH-018-Br (0.01-1 mg/kg) or $\Delta^9$-THC (0.1-3 mg/kg) affected the distance travelled (fig. 3), the average speed (fig. 1-S) and the immobility time (fig. 2-S) in mice during the choice phase performed at 2 hrs, but not at 24 hrs.

*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/kg (~ 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). $\Delta^9$-THC reduced the total distance travelled by mice only at 3 mg/kg (~ 38%; $F_{4,49}$= 11.33; p<0.0001; fig. 3-D). Treatment with AM 251 (1 mg/kg) completely prevented motor changes induced by JWH-018-R compounds and $\Delta^9$-THC [significant effect of agonists ($F_{4,90}$=2.744, p=0.0332), AM 251 ($F_{1,90}$=38.21, p<0.0001) and agonist x AM 251 interaction ($F_{4,90}$=2.519, p=0.0467); fig. 3-E].

*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 average speed in mice ($F_{(4,49)}$= 10.27; p<0.0001; fig. 1S-A). On the other hand, JWH-018-Cl reduced (~ 71%) the average speed in mice ($F_{4,49}$= 4.478; p=0.0039; fig. 1S-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/kg, respectively ($F_{4,49}$= 6.259; p=0.0004; fig. 1S-C). $\Delta^9$-THC did not affect this parameter in the NOR test (fig. 1S-D). Treatment with AM 251 (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), AM 251 ($F_{1,90}$=27.18, p<0.0001) and agonist x AM 251 interaction ($F_{4,90}$=2.552, p=0.0444); fig. 1S-E].

*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 immobility time in mice ($F_{4,49}$= 8.529; p<0.0001; fig. 2S-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. 2S-B; JWH-018-Br: ~ 202%; $F_{4,49}$= 2.857; p=0.0342; fig. 2S-C). $\Delta^9$-THC increased the immobility time in mice at 3 mg/kg (~ 196%; $F_{4,49}$= 2.778; p=0.038; fig. 2S-D). Treatment with AM 251 (1 mg/kg) completely prevented the motor changes induced by JWH-018-R compounds and $\Delta^9$-THC [significant effect of agonists ($F_{4,90}$=3.665, p=0.0082), AM 251 ($F_{1,90}$=29.07, p<0.0001) and agonist x AM 251 interaction ($F_{4,90}$=2.6, p=0.0413); fig. 2S-E].

### 3.2. Tail suspension test.

The TS test was used to investigate the effect of JWH-018-R compounds and $\Delta^9$-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 $\Delta^9$-THC (0.1-3 mg/kg) increased the immobility time in the TS test both at 2 hrs and 24 hrs (fig. 4).

There were no significant differences in the immobility time between untreated control animals and vehicle-treated mice (2 hrs after vehicle administration: t=0.1718, df=18, p=0.8655; 24 hrs: t=0.1407, df=18, p=0.8897; *data not shown*). JWH-018 significantly increased the immobility time at 2 hrs ($F_{4,49}$= 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 hrs ($F_{4,49}$= 16.46; p<0.0001 fig. 4-A). JWH-018-Cl increased the immobility time both at 2 hrs (~ 189%; $F_{4,49}$= 8.72; p<0.0001) and 24 hrs (~ 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 hrs ($F_{4,49}$= 12.14; p<0.0001) at 0.3 mg/kg (~ 154%) and 1 mg/kg (~ 227%) and these effects persisted at 24 hrs ($F_{4,49}$= 16.86; p<0.0001 fig. 4C). The administration of $\Delta^9$-THC

significantly increased the immobility time at 2 hrs ($F_{4,49}$= 4.633; p=0.0032) only at 3 mg/kg (~ 163%) and the effect persisted at 24 hrs (~ 173%; $F_{4,49}$= 11.83; p<0.0001; fig. 4-D). Treatment with AM 251 (1 mg/kg) completely prevented the effect of JWH-018-R compounds and $\Delta^9$-THC on the TS test both at 2 hrs [significant effect of agonists ($F_{4,90}$=4.964, p=0.0012), AM 251 ($F_{1,90}$=51.83, p<0.0001) and agonist x AM 251 interaction ($F_{4,90}$=5.141, p=0.0009] and 24 hrs [significant effect of agonists ($F_{4,90}$=3.093, p=0.0196), AM 251 ($F_{1,90}$=39.16, p<0.0001) and agonist x AM 251 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 $\Delta^9$-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. 5A as of time points during the course of the experiments. JWH-018 induced a concentration-dependent depressive effect on fEPSP (fig. 5-B; n=5-6). In particular, fEPSP amplitude resulted modified 3 to 5 minutes after the onset of the perfusion with JWH-018 (1 µM) containing aCSF. In most experiments JWH-018 induced a progressive depressing effect, reaching a steady state after 40/45 minutes. In 4 experiments where it was present at the maximal dose (1 µM), this depression was preceded by a short (2-5 minutes) period of increased fEPSP , whose maximal amplitude changed greatly from test to test, ranging from approximately +10 up to +50% of control. After this phase, the slope value declined progressively depression previously described about the other experiments (53.5% ± 17.9 depression, n=13). This different response to JWH-018 had the effect to expand the variability of fEPSP values in the first minutes after the treatment. For comparison, fig. 5-A also reported the effect of vehicle on fEPSP slope (101.3% ±10.1, n=6). 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 (1µM), 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, $\Delta^9$-THC (1 µM) was ineffective on fEPSP amplitude (n=2; fig. 5-B). To verify the receptor selectivity of JWH-018 (1µM), tests (n=3) have been conducted by adding the $CB_1$ receptor antagonist AM 251 (2 µM) to the perfusing solution 30 min before JWH-018 and throughout the entire experiment. The antagonist by itself did not modified fEPSP (*data not shown*), while it blocked the JWH-018-induced effects (n=2; fig. 5-A).

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. In the presence of steady effect of JWH-018 (1μM), 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 (n=5; fig. 5-D). A similar effect was induced by JWH-018-Cl and JWH-018-Br (1μM; n=4 and n=3, respectively). When clearly detectable, the fiber volley amplitude has been measured, by comparing the effect of JWH-018-R compounds (1μM) to that of vehicle (fig. 1-E). At steady state of fEPSP, a non-significant trend to a reduction in fiber volley was observed.

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 (1μM) treatment. When compared to the vehicle (n=6), JWH-018 (n=13) almost completely blocked the development of early and late LTP, impairing the formation of stable potentiation. Similar results were produced by JWH-018-Br and JWH-018-Cl (1 μM; n=4 and n=3, 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, it was impossible to build up any further stable potentiation, accounting for a consistent LTP inhibitory effect (*data not shown*).

3.3.4. Effect of JWH-018-R compounds on glutamate and GABA release in hippocampal slices.
*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 and 7-B). The addition of JWH-018 (0.1 and 1 μM), JWH-018-Cl (1μM) and -Br (1μM) to the perfusion medium did not significantly affect spontaneous glutamate and GABA release from rat hippocampus slices (fig. 7-A and 7-B).

*$K^+$-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 observed 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 lower concentration (0.1 μM), JWH-018 did not significantly affect $K^+$-evoked glutamate and GABA release (fig. 7-C and 7-D). To verify the receptor selectivity of JWH-018, the experiments have been repeated in the presence of AM 251. As shown in fig. 7-C and 7-D, when AM 251 (2 μM) was added to the perfusion solution 10 min before JWH-018 (1 μM), it completely blocked the effects of the agonist. By itself, AM 251 (2 μM) did not affect $K^+$-evoked glutamate and GABA release (*data not shown*).

Finally, similarly to JWH-018, JWH-018-Br (1 μM) and JWH-018-Cl (1 μM) significantly decreased $K^+$-evoked glutamate and GABA release (fig. 7-C and 7-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 $\Delta^9$-THC impair short (2 hrs) and long term (24 hrs) 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 AM 251 (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 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; 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; Clarke et al., 2008; 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 hrs) and long term (24 hrs) 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 mouse motivation to move during 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 reduce the motivation of the mouse to react to an unfavorable situation as reproduced in the TS test. However, this “demotivational state” is induced at a dose (0.3 mg/kg) that impaired the memory in mice without to depress motor activity and the TOE time. This suggests that memory

impairment caused by JWH-018-R compounds up to 0.3 mg/kg is independent from the demotivation or anhedonia of mice. 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 hrs, might be due to a reduction in the locomotion of mice. However, this is not the case of the cognitive impairments observed 24 hrs 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), $\Delta^9$-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 hrs after JWH-018 administration (*personal unpublished data*), while the working memory impairment induced by the compound was still detectable after 24 hrs after its administration. Moreover, JWH-018 (0.1 mg/kg) did not affect the total object exploration time. Taken together, these data suggest that the cognitive deficit observed in NOR test is 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) suggests a drug-induced impairment in the already-acquired memory (Ennaceur, 2010). However, it cannot be ruled out that this effect could be related to the rewarding properties of synthetic cannabinoids (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 hrs after its administration, being this effect no more detectable after 24 hours. 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 hrs.

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 $\Delta^9$-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., 2011; 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 most of the experiments with the highest concentration of JWH-018 tested (1 μM), a relatively short transient increase of the fEPSP, characterized by a large variability both in amplitude and time required to extinction, has also been observed.

Our data show that JWH-018-R compounds act at presynaptic level, as suggested by the observed significant selective reduction of paired pulse facilitation ratio. Furthermore, JWH-018-R compounds reduce fiber volley amplitude in marginal extent, ruling out this parameter as a possible responsible of the fEPSP depression. The $CB_1$ receptor selectivity of JWH-018 has been confirmed by the selective $CB_1$ antagonist AM 251, which completely blocked the effects previously described. The *in vitro* experiments also show that in the same preparation, JWH-018-R compounds (1 μM) almost completely suppressed the electrically induced LTP. As even lower concentrations exerted a highly significant LTP inhibition but a minimal depression of fEPSP, the compound-induced effects on LTP could be, almost in part, disclosed from fEPSP amplitude. Previous findings demonstrated that repeated exposure to $\Delta^9$-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, Sullivan 1999, Hajos 2001; Mereu 2003; Diana et al., 2003; Domenici et al., 2006; Nemeth 2008; Bajo 2009; Roloff 2009; Ledgerwood 2010; Peterfi 2012) and GABAergic (Hoffman and Lupica, 2000; Hajos 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 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 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 EPSP 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, $\Delta^9$-THC (1 μM) failed to affect fEPSP. This is in contrast with the results reported by (Hoffman et al., 2016) where the same $\Delta^9$-THC concentration exerted a clear inhibitory effect. This difference could be possibly due to the different vehicle used to solubilize $\Delta^9$-THC in the present experiments (EtOH) and in the study by Hoffman et al. (DMSO), coupled to the partial agonist activity of $\Delta^9$-THC at $CB_1$ receptors (Laaris et al., 2010). In fact, EtOH exerts an occluding effect (reff), 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 adenosine A1 receptor antagonist to block the endogenous adenosine reducing effects on $CB_1$ receptor-mediated inhibition of glutamate release in the hippocampus (Hoffman et al. 2010). This could also explain the differences in $\Delta^9$-THC effects and in JWH-018 sensitivity between their and present experiments.

In the present study, the early effect of JWH-018 (1 μM) is a transient but well detectable hyperexcitability. This effect is unique for the parent compounds, as it is not displayed by JWH-018-Cl or JWH-018-Br. This could be attributed to a $CB_1$ receptor-presynaptic activation on GABA terminals, revealing for an immediate access of JWH-018 to the slice core, followed by a slower developing of the depressive effect on fEPSP. The presence of active cannabinoid receptors on hippocampal GABA terminals of interneurons has been documented (Hajos 2000; Ferraro 2002; Trettel and Levine, 2002; Chevaleyre and Castillo, 2003; Peterfi et al., 2012; Laaris et al., 2010), but the net activity of JWH-018 on GABA presynaptic $CB_1$ receptors still needs to be quantified. 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 the present, the JWH-018-induced time discrepancy between GABA and glutamate inhibition, observed in the

electrophysiological experiments, is difficult to be explained. 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 CB1 receptors JWH-018 activates ERK1/2 MAPK, with a typical rapid time course (peak in 5/10 min). This rapid activation is straight forward linked to GABA currents, having 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 amigdala, where $CB_1$ receptors presence and activity has been demonstrated (Aj-Dahmane 2009; Azad 2003-2008; Domenici et al., 2006; Irie et al., 2015).

The paired pulse facilitation results are aligned with the expected effects of a $CB_1$ receptor agonist (Atwood et al., 2010; Atwood et al., 2011), with a clear indication of a presynaptic mechanism involving the reduction of neurotransmitter release (Shen et al., 1996).

Similarly to other CB1 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. In the present study, late-LTP induction was antagonized in a concentration-dependent way. JWH-018 was applied 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 (ref). Although JWH-018 interferes with $CB_1$ receptors with high affinity (Aung et al., 2000; Jarbe 2011), 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. Further experiments are necessary to elucidate this aspect. (Robinson, 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 $\Delta^9$-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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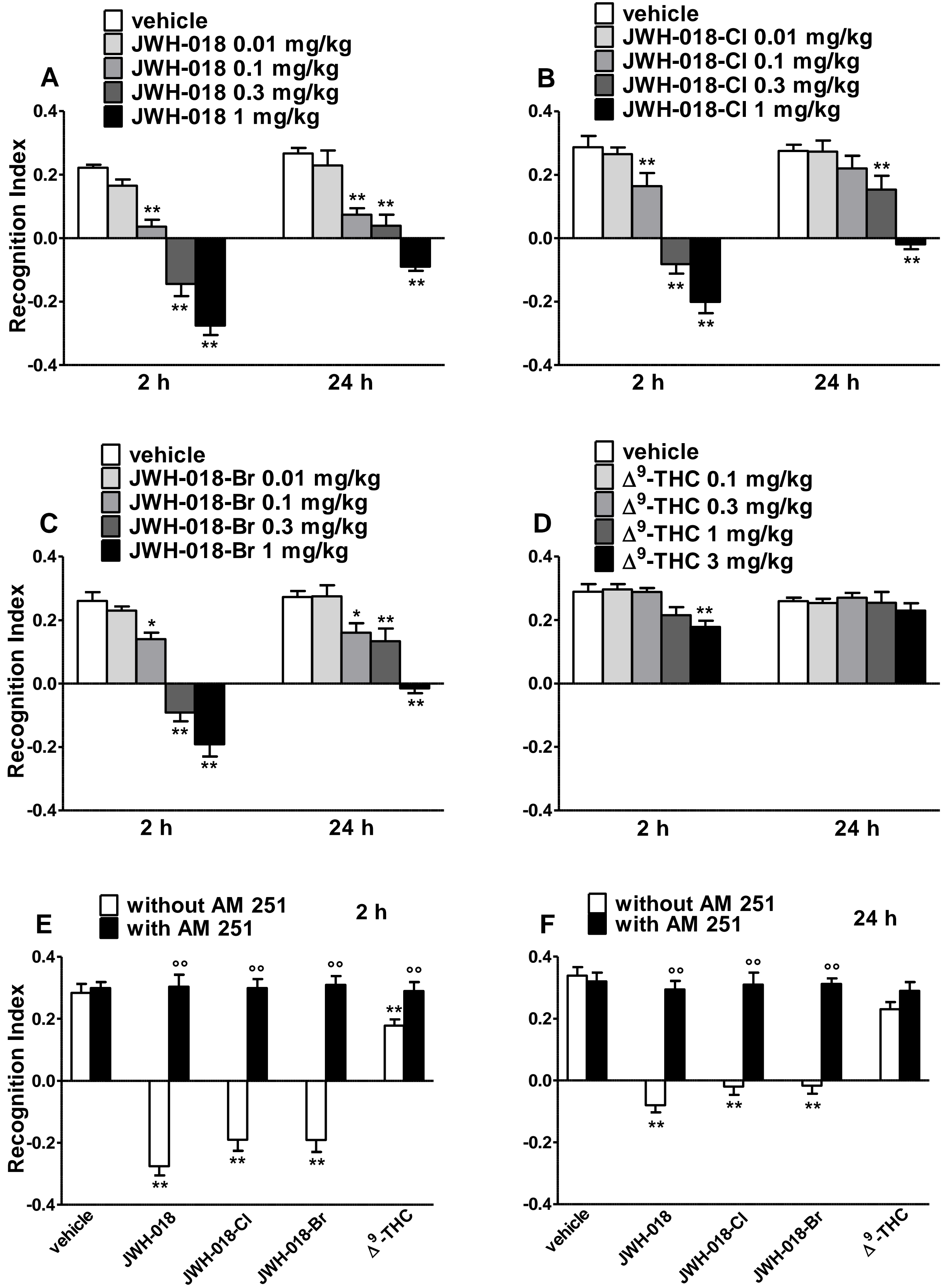


Figure 1

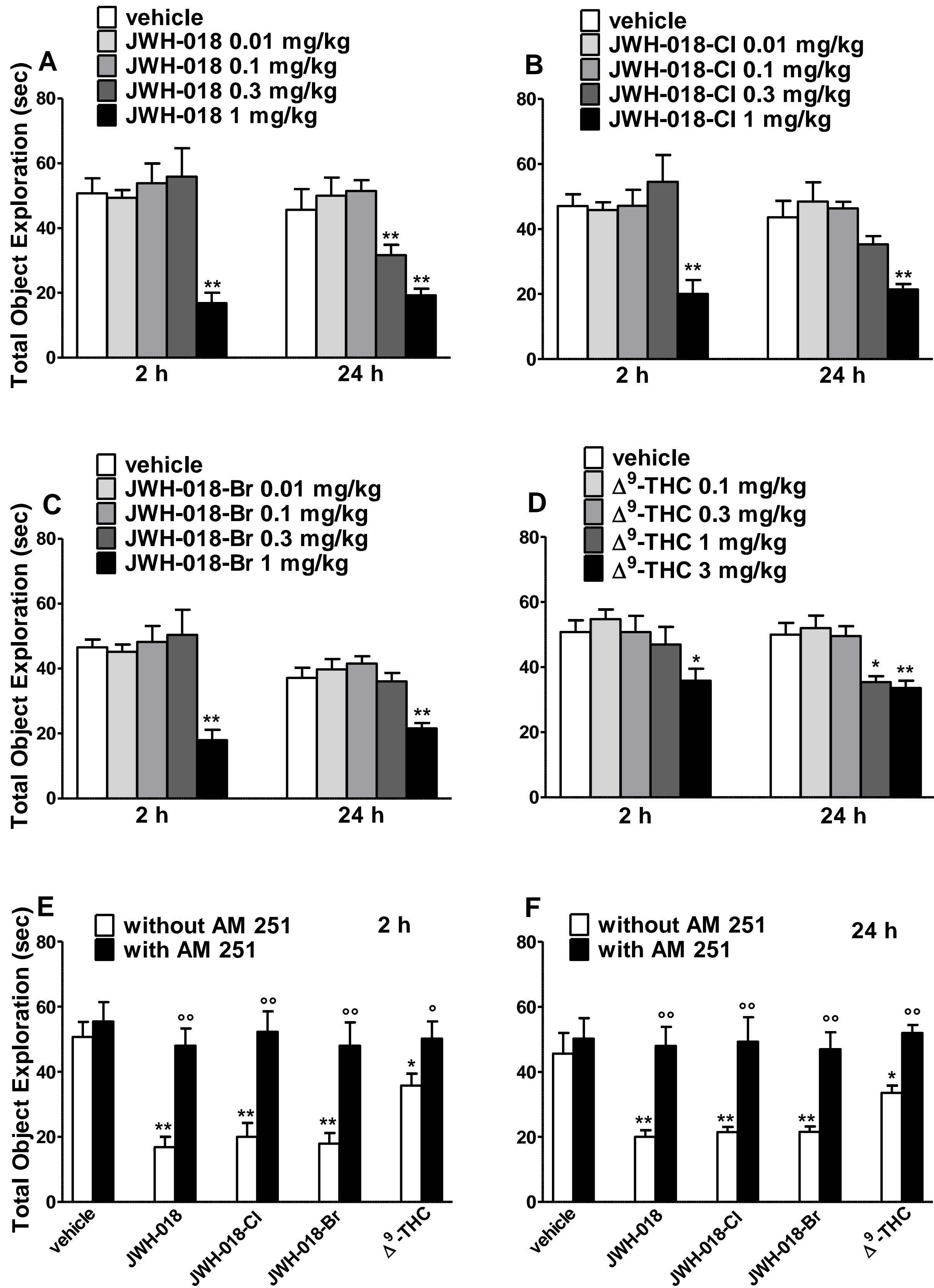


Figure 2

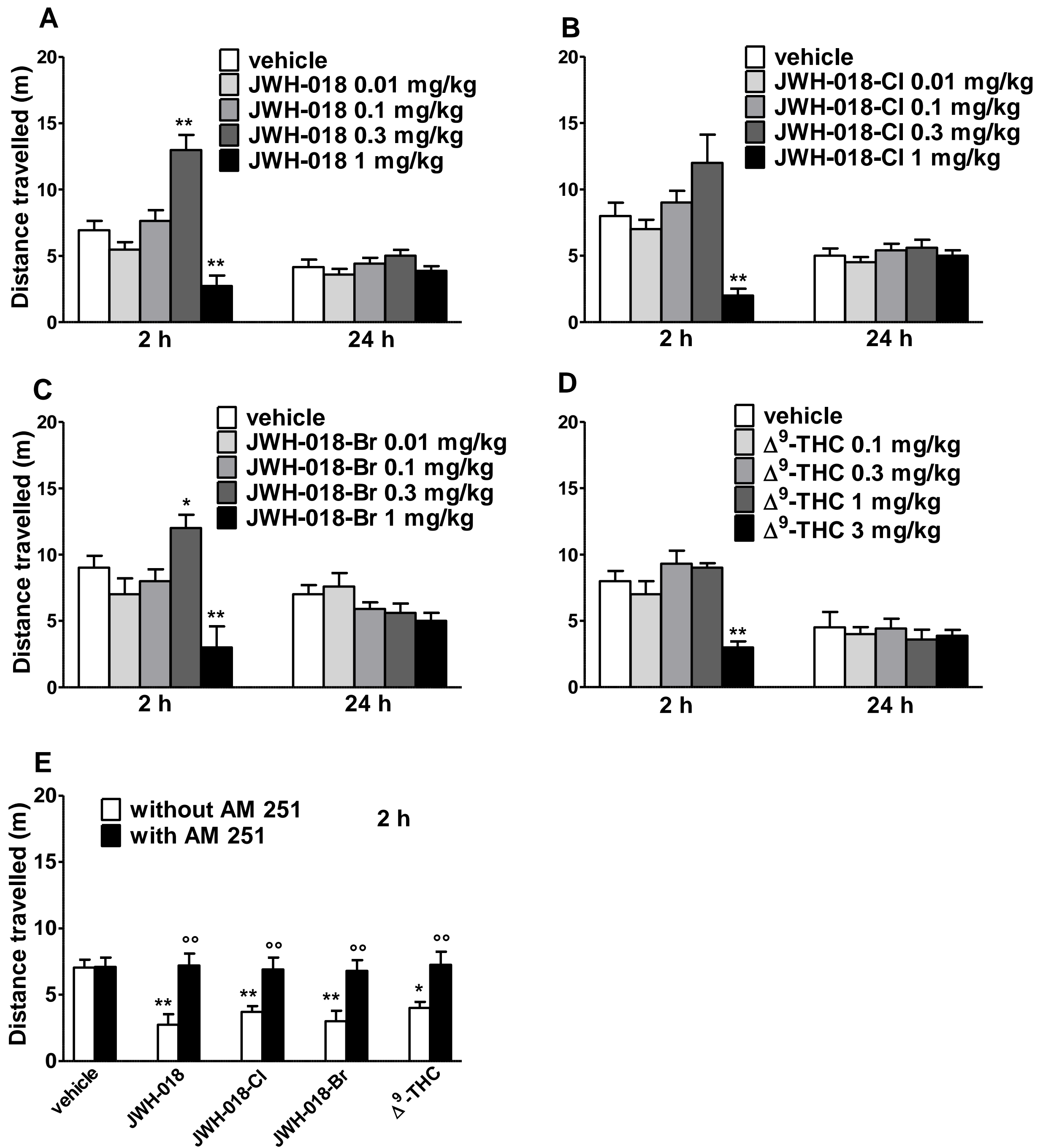


Figure 3

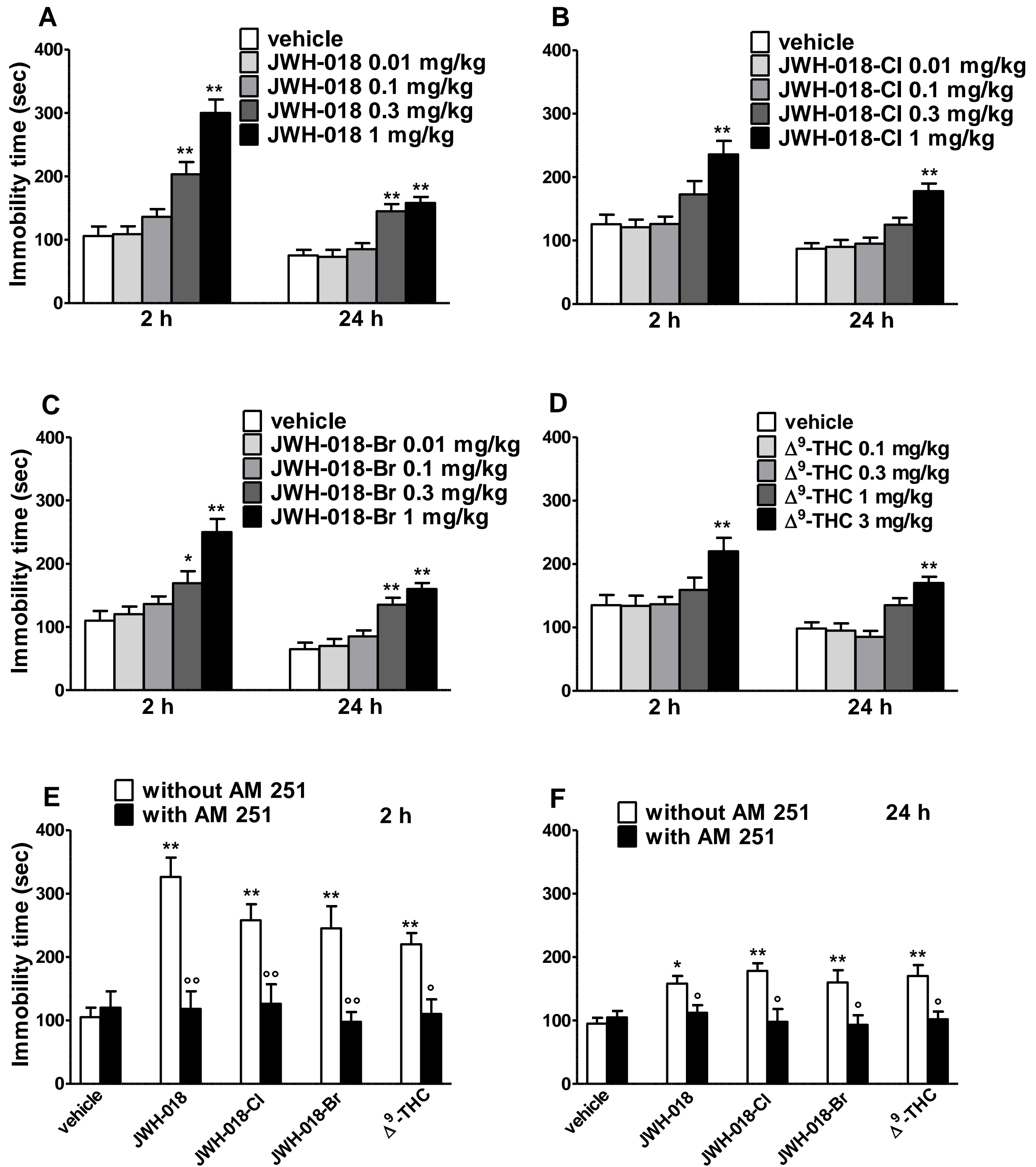


Figure 4

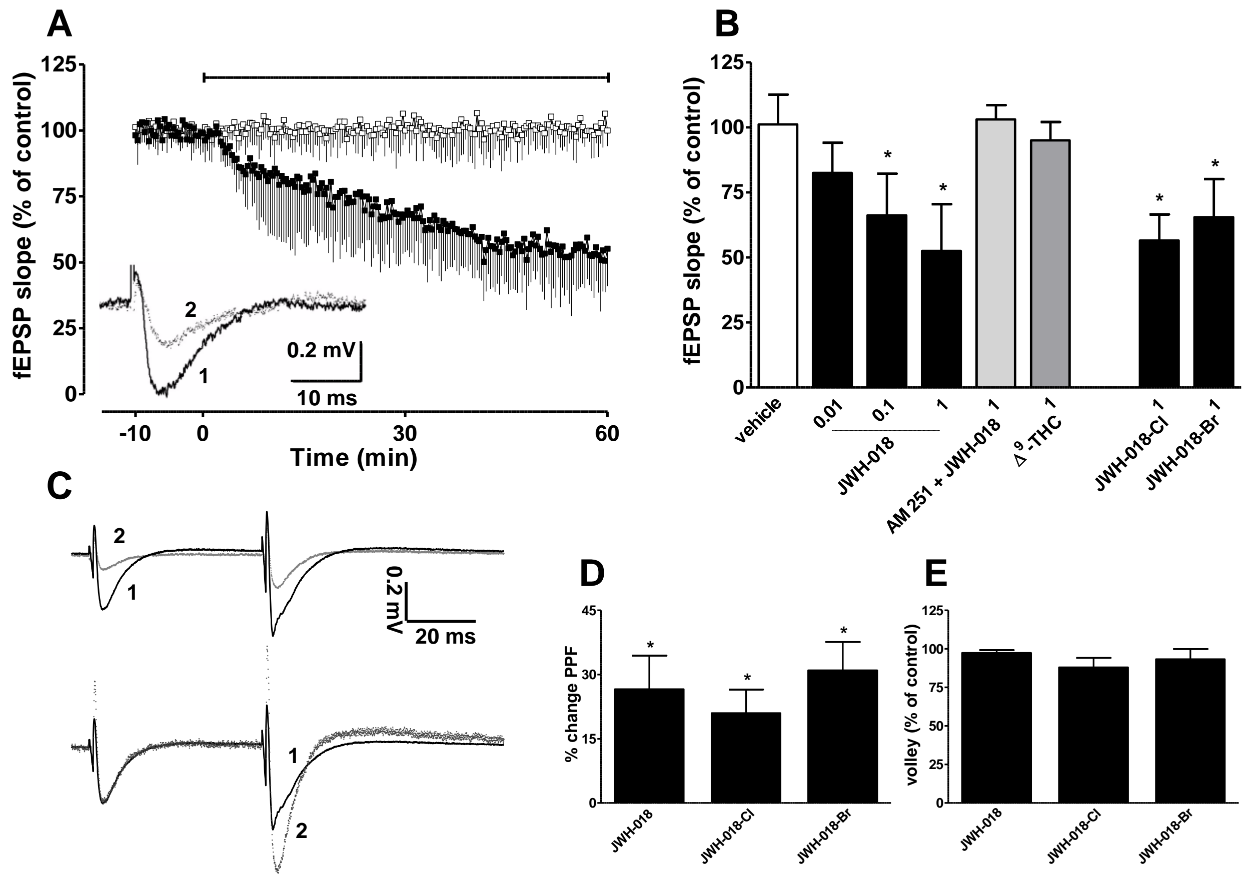


Figure 5

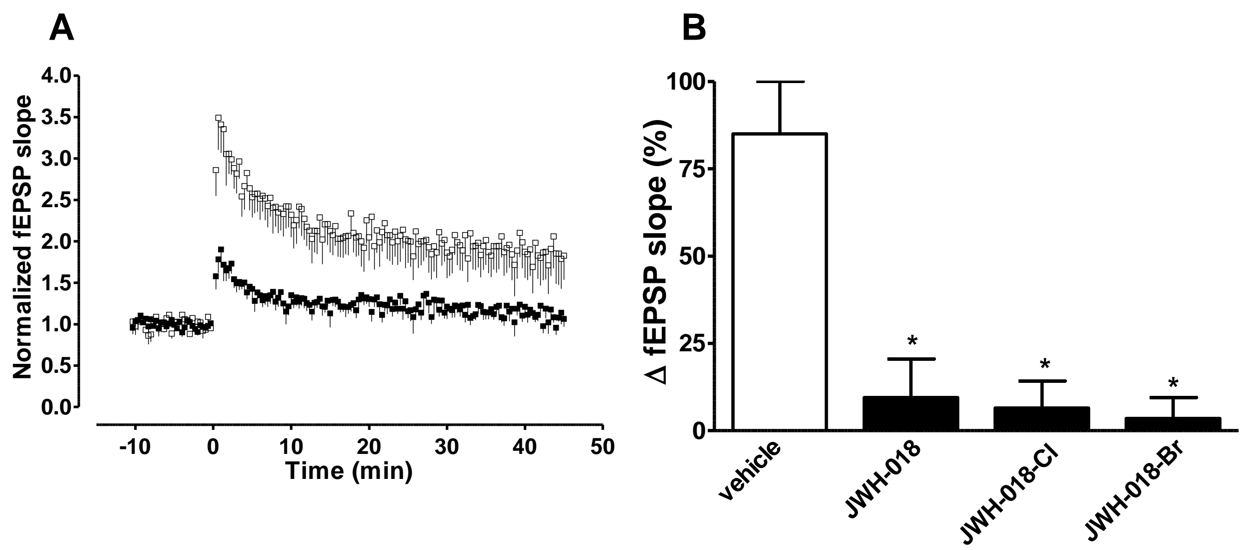


Figure 6

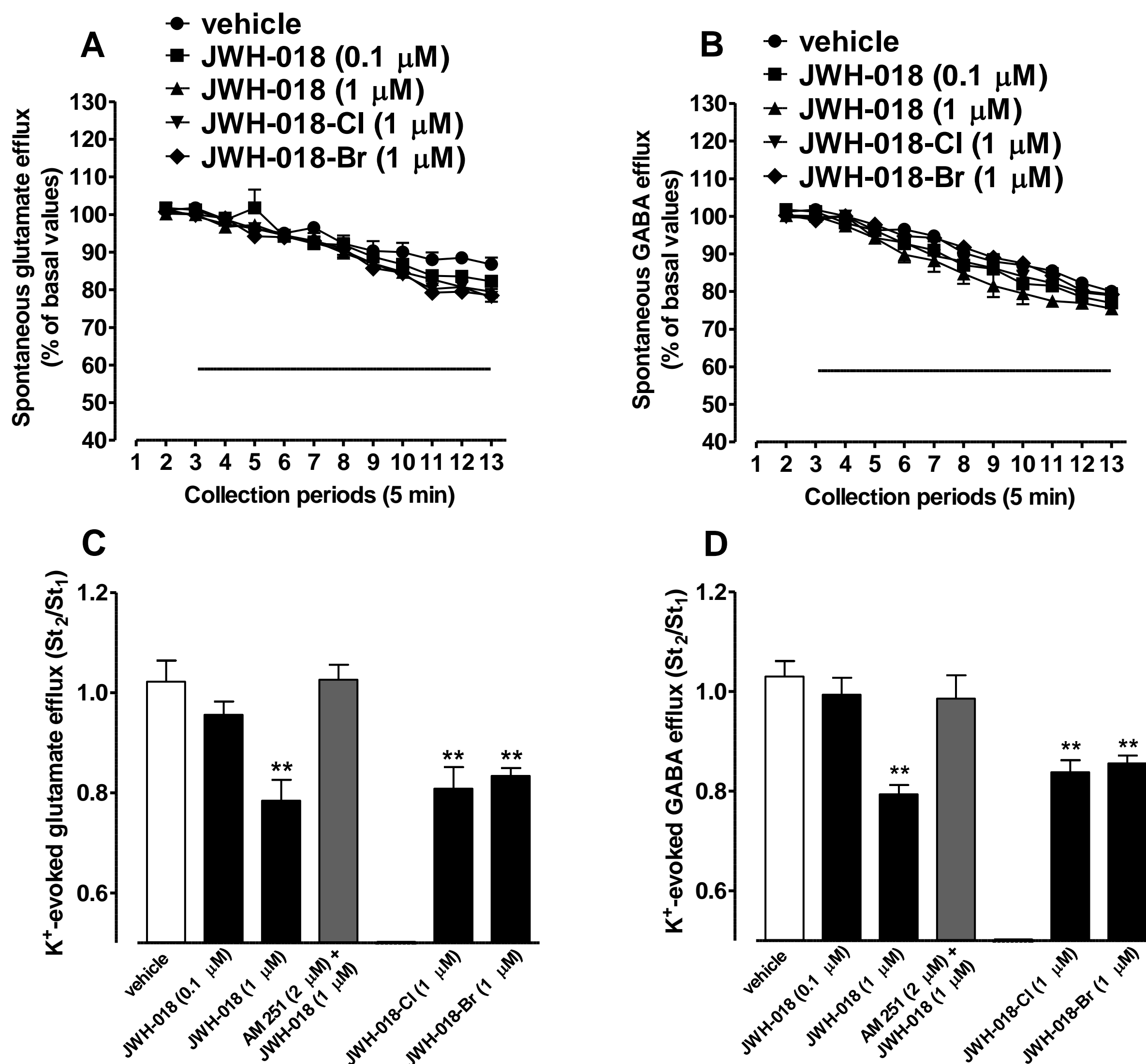


Figure 7

# Figure legends

**Figure 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 Δ9-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 $\Delta^9$-THC given 15 mins after the familiarization phase impaired the short- (at 2 hrs) and long- (24 hrs) memory recognition in mice. AM 251 (1 mg/kg i.p.) administered 20 mins before agonists prevented the impairment of the RI both at 2 hrs (panel E) and 24 hrs (panel F). Data are expressed as Recognition Index (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 AM 251 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.

**Figure 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 Δ9-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 $\Delta^9$-THC given 15 mins after the familiarization phase impaired the TOE both at 2 and 24 hrs. AM 251 (1 mg/kg i.p.) administered 20 mins before agonists prevented the impairment induced by cannabinoid agonists both at 2 hrs (panel E) and 24 hrs (panel F). 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 AM 251 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.

**Figure 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 Δ9-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 $\Delta^9$-THC given 15 mins after the familiarization phase affected the distance travelled at 2 hrs. AM 251 (1 mg/kg i.p.) administered 20 mins before agonists prevented the impairment induced by cannabinoid agonists (panel E). 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 AM 251 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.

**Figure 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 $\Delta^9$-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 $\Delta^9$-THC given 15 mins after the familiarization phase increased the immobility time both at 2 and 24 hrs. AM 251 (1 mg/kg i.p.) administered 20 mins before agonists prevented the impairment in the TS both at 2 (panel E) and 24 hrs (panel F). 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 AM 251 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.

**Figura 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 part of the graph. Hollow dots correspond to control/vehicle conditions (n=8), black dots corresponds to JWH018 1μM (n=13). Inset shows two typical fEPSP recorded during the same experiment: control (vehicle) (5 minutes before JWH108 application) = [1] - black line, 1 μM JWH018 (45 min application) = [2] - dotted line. **(B)** Histogram reporting peak values at steady state, as average fEPSP slope of last 3 minutes of recording. Values corresponds to % changes respect to control condition (average values of fEPSP slope 10 minutes before drug application). First line of X axis, is drug concentration (μM) in line two are the corresponding drugs applied. For brevity, -Cl and –Br stays for JWH018-Cl and JWH018-Br. When AM251 was applied, JWH018 1μM was the test drug and concentration. n= (CTL=13, JWH018 0.01 mM= 6, JWH018 0.1 mM=5, JWH018 1 mM=13, JWH018-Cl=3, JWH018-Br=4, AM251+JWH018=2, Δ9-THC =3). **(C)** Representative superimposed traces recorded applying the Paired Pulse Facilitation PPF paradigm. Traces are relative to the same experiment. Upper couple of traces refers to: [1] recording in control (vehicle), [2] after 45 min. JWH018 1μM. Lower traces (SCALED) are same as upper but after normalization at first stimulus, to improve comparison of PPF effect on second stimulus response. **(D)** Maximal effect on paired pulse facilitation (PPF) at steady state of JWH018, -Cl, -Br (1μM). n= (JWH018=5, JWH018-Cl=3, JWH018-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%. **(E)** Maximal effect at steady state of JWH018, -Cl, -Br, on fiber volley amplitude calculated as % of amplitude before drug application. n= (JWH018=11, JWH018-Cl=3, JWH018-Br=4). Error bars = s.d. values. * =p>0.05 *vs*. control.

**Figure 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 1μM JWH018 (n=13). Error bars corresponds to s.d. of corresponding averaged value. **(B)** Histogram comparing averaged maximal effect on fEPSP after TB5 stimulation at steady slope, in control condition (hollow bar, n=6) and after JWH018 (n=13), JWH018-Cl (n=3) and JWH018-Br (n=4), all at 1μM (black bars). Error bars = s.d. *=p>0.01 (vehicle vs. drug).

**Figure 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. 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. $^{**}p<0.01$ significantly different from the respective vehicle group according to ANOVA followed by Newman-Keuls test for multiple comparisons.

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**Supplementary Materials**

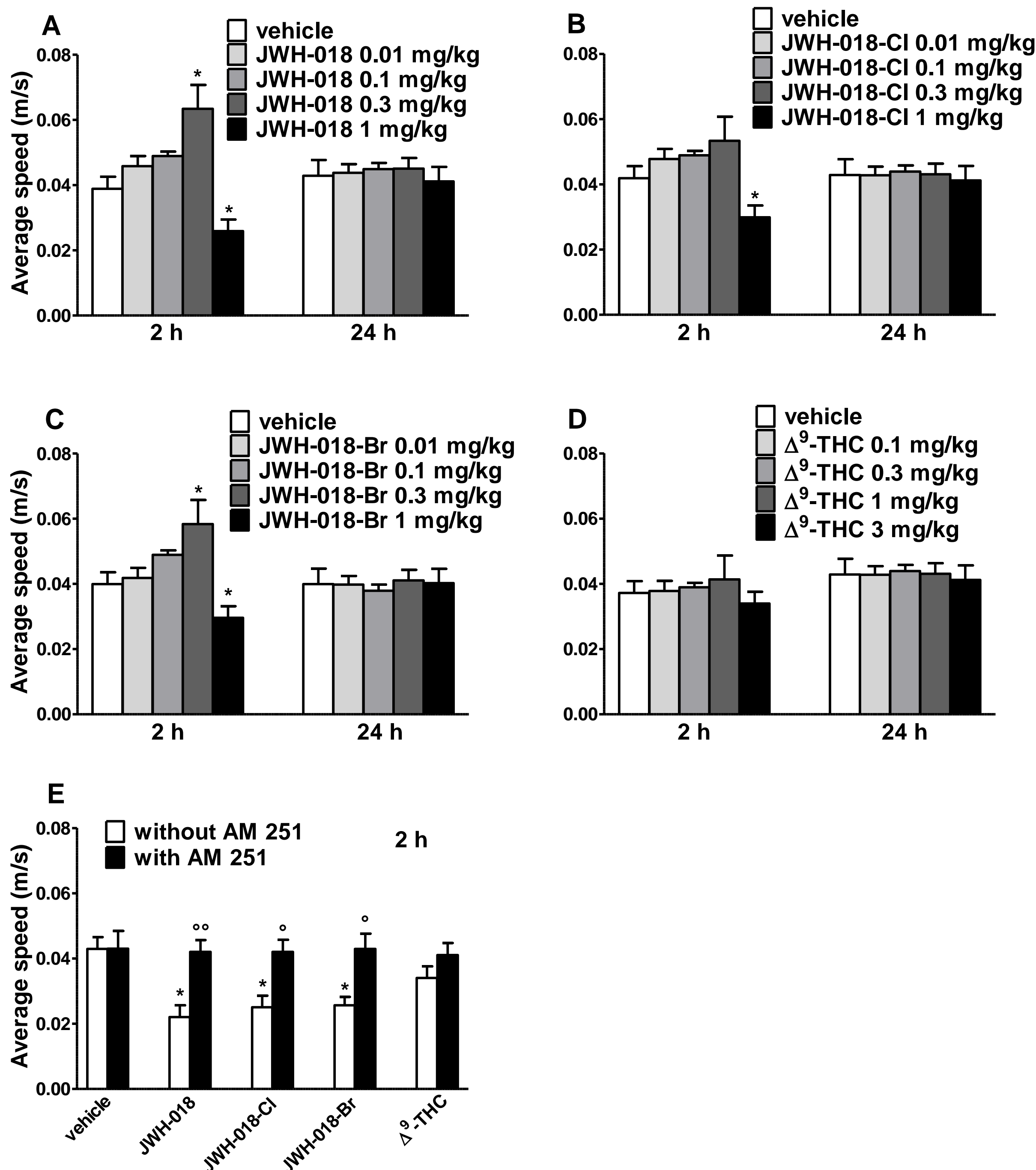


**Figure 1S.** Effect of systemic administration (0.01-1 mg/kg i.p.) of JWH-018 (panel A), JWH-018-Cl (panel B), JWH-018-Br (panel C) and Δ9-THC (0.1-3 mg/kg i.p.; panel D) on the average speed in the NOR test in mice. JWH-018, JWH-018-Cl, JWH-018-Br and $\Delta^9$-THC given 15 mins after the familiarization phase affected the average speed at 2 hrs. AM 251 (1 mg/kg i.p.) administered 20 mins before agonists prevented the impairment induced by cannabinoid agonists (panel E). Data are expressed as absolute values (m/s) 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 AM 251 was performed by two-way ANOVA followed by the Bonferroni's test. Panel A: significant effect of treatment at 2 hrs ($F_{(4,49)}$= 10.27; P<0.0001). Panel B: significant effect of treatment at 2 hrs ($F_{(4,49)}$= 4.478; P=0.0039). Panel C: significant effect of treatment at 2 hrs ($F_{(4,49)}$= 6.259; P=0.0004). Panel E: significant effect of agonists ($F_{4,90}$=2.735, p=0.0337), AM 251 ($F_{1,90}$=27.18, p<0.0001) and agonist x AM 251 interaction ($F_{4,90}$=2.552, p=0.0444). *p<0.05 versus vehicle. °°p<0.01, °p<0.05 versus agonist administration.

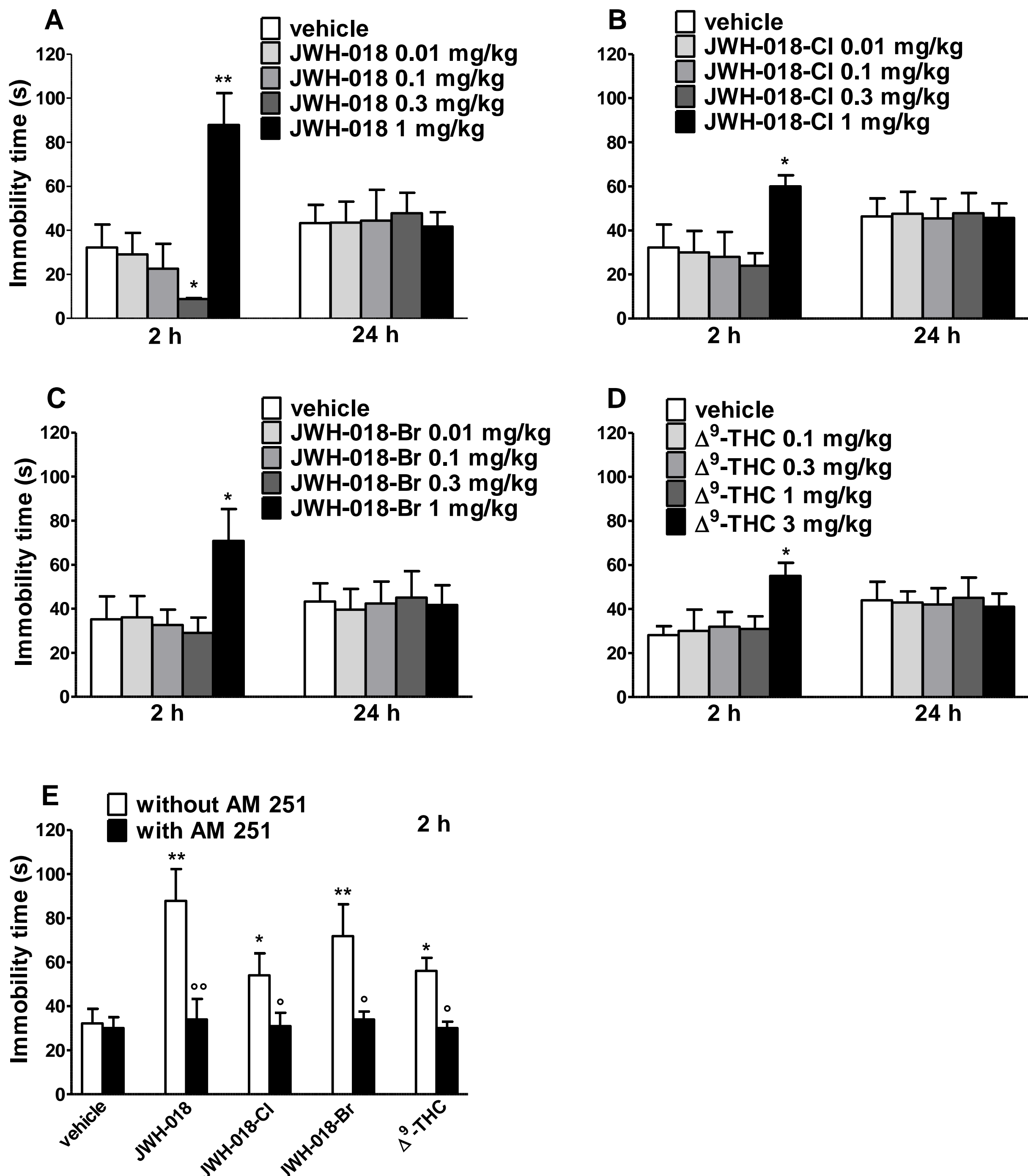

**Figure 2S.** Effect of systemic administration (0.01-1 mg/kg i.p.) of JWH-018 (panel A), JWH-018-Cl (panel B), JWH-018-Br (panel C) and Δ9-THC (0.1-3 mg/kg i.p.; panel D) on the immobility time in the NOR test in mice. JWH-018, JWH-018-Cl, JWH-018-Br and $\Delta^9$-THC given 15 mins after the familiarization phase affected the distance travelled at 2 hrs. AM 251 (1 mg/kg i.p.) administered 20 mins before agonists prevented the impairment induced by cannabinoid agonists (panel E). 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 AM 251 was performed by two-way ANOVA followed by the Bonferroni's test. Panel A: significant effect of treatment at 2 hrs ($F_{(4,49)}$= 8.529; P<0.0001). Panel B: significant effect of treatment at 2 hrs ($F_{(4,49)}$= 2.678; P=0.0437). Panel C: significant effect of treatment at 2 hrs ($F_{(4,49)}$= 2.857; P=0.0342). Panel D: significant effect of treatment at 2 hrs ($F_{(4,49)}$= 2.778; P=0.038). Panel E: significant effect of agonists ($F_{4,90}$=3.665, p=0.0082), AM 251 ($F_{1,90}$=29.07, p<0.0001) and agonist x AM 251 interaction ($F_{4,90}$=2.6, p=0.0413). **p<0.01, *p<0.05 versus vehicle. °°p<0.01, °p<0.05 versus agonist administration.