Effect of the novel synthetic cannabinoids AKB48 and 5F-AKB48 on "tetrad", sensorimotor, neurological and neurochemical responses in mice. In vitro and in vivo pharmacological studies.

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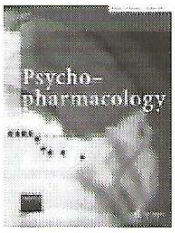

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Effect of the novel synthetic cannabinoids AKB48 and 5F-AKB48 on "tetrad", sensorimotor, neurological and neurochemical responses in mice. In vitro and in vivo pharmacological studies.

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Conflict of interest

The authors have no conflict of interest to declare.

Abbreviations

AM 251	1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide
AKB48	N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide
DA	dopamine
NAc shell	Nucleus Accumbens shell
Δ^9-THC	(-)-Δ^9-THC or Dronabinol®
JWH-018	Naphthalen-1-yl-(1-pentylindol-3-yl)methanone
5F-AKB48	N-(1-adamantyl)-1-(5-fluoropentyl)-1H-indazole-3- carboxamide

Abstract

Rationale. AKB48 and its fluorinate derivate 5F-AKB48 are two novel synthetic cannabinoids belonging to a structural class with an indazole core structure. They are illegally marketed as incense, herbal preparations or chemical supply for theirs psychoactive Cannabis-like effects.

Objectives. The present study was aimed at investigating the in vitro and in vivo pharmacological activity of AKB48 and 5F-AKB48 in male CD-1 mice and to compare their in vivo effects with those caused by the administration of Δ^9-THC and JWH-018.

Results. In vitro competition binding experiments performed on mouse and human CB_1 and CB_2 receptors revealed a nanomolar affinity and potency of the AKB48 and 5F-AKB48. In vivo studies showed that AKB48 and 5F-AKB48, induced hypothermia, increased pain threshold to both noxious mechanical and thermal stimuli, caused catalepsy, reduced motor activity, impaired sensorimotor responses (visual, acoustic and tactile), caused seizures, myoclonia, hyperreflexia and promoted aggressiveness in mice. Moreover, microdialysis study in freely moving mice showed that systemic administration of AKB48 and 5F-AKB48 stimulated dopamine release in the nucleus accumbens. Behavioral, neurological and neurochemical effects were fully prevented by the selective CB_1 receptor antagonist/inverse agonist AM 251.

Conclusions. For the first time the present study demonstrates the overall pharmacological effects induced by the administration of AKB48 and 5F-AKB48 in mice and suggests that the fluorination can increase the power and/or effectiveness of SCBs. Furthermore, this study outlines the potential detrimental effects of SCBs on human health.

Keywords: AKB48; 5F-AKB48; JWH-018; Δ^9-THC; sensorimotor responses; cannabinoids; synthetic cannabinoids; behavior; microdialysis.

1. Introduction

During the first half of 2013, AKB48 (N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide) and its derivative fluorinated 5F-AKB48 (N-(adamantan-1-yl)-1-(4-fluorobutyl)-1H-indazole-3-carboxamide) formed respectively 1.0 and 2.5% of all synthetic cannabinoids (SCBs) reported by the DEA-operated National Forensic Laboratory Information System in the USA (NFLIS 2013). Toxicological and forensic analysis revealed their presence in seized products or in biological fluids of people subjected to toxicological control (Uchiyama et al. 2012; Karinen et al. 2015; Vikingsson et al. 2015; Odoardi et al. 2016). As well described by Santacroce and collaborators (Santacroce et al. 2015), AKB-48 and 5FAKB48 may be retrieved in products sold as incense mixtures, as a sole ingredient infused on herbs or as a powder (EMCDDA 2009; EMCDDA 2015). AKB48 and 5F-AKB48 may be added to tobacco or sprayed on leaves and then smoked, inhaled from heated aluminum foil, dissolved in ethanol and finally ingested with lipid-rich foods or vaporized (DrugsForum 2012a; DrugsForum 2012b). AKB48 and 5F-AKB48 bind at nanomolar concentration at CB_1 and CB_2 cannabinoid receptor (Uchiyama et al. 2013; De Luca et al. 2015b) suggesting that they could have similar or more higher in vivo effects as others SCBs. Recent findings showed that the adamantylindazole compounds (i.e. 5F-AKB48) induce DNA-damage at the chromosomal level, without cause gene mutations (Koller et al. 2015). AKB48 and 5F-AKB48 do not belong to any of the seven groups commonly used to classify synthetic cannabinoids: cyclohexylphenol (such as cannabicyclohexanol (CCH) and CP-47497), classical cannabinoids (such as HU-210), naphthoylindoles (such as JWH-018 and JWH-073), phenylacetylindoles (such as JWH-250 and JWH-203), benzoylindoles (such as AM-694 and RCS-4), naphthoylnaphthalenes (such as CB-13) and adamantylindoles (APICA) but are adamantylindazole (Uchiyama et al. 2012). In particular, AKB48 (also known as APINACA) differs from earlier JWH-type SCBs by having an adamantyl group connected to an indazole moiety through a carboxamide linkage. Furthermore, to increase the lipophilicity of AKB48, hence enhancing the absorption through biological membranes/blood brain barrier (Schifano et al. 2015), a fluorine atom was linked at the 5-pentyl position of the indazole scaffold. This formulation strategy was previously carried out for AM-2201, the fluorinated analog of JWH-018 (Ghandi et al. 2013). The metabolism of AKB48 and 5F-AKB48 has been identified using a hepatocyte model (Ghandi et al. 2013) and human liver microsomal incubation (Holm et al. 2015). In particular, AKB48 was metabolized in 11 major metabolites that included monohydroxylated, dihydroxylated, trihydroxylated, and mono- and dihydroxylated glucuronide conjugates and dihydroxylated with ketone formation at the N-pentyl side chain (Ghandi et al. 2013). As reported for others SCBs, this aspect should be considered since, a large number of metabolites could maintain agonistic activity at CB_1 receptors, as demonstrated for JWH-018 and

other SCBs (Brents et al. 2011; Brents et al. 2012). Despite the presence of these in vitro metabolism studies, there are poor preclinical in vivo evidences on pharmaco-toxicological effects of these SCBs. Recently, it was shown that 5F-AKB48 facilitated dopamine (DA) release in the Nucleus Accumbens shell of rats (De Luca et al. 2015b), suggesting its potential positive role in rewarding mechanisms (Miliano et al. 2016), as already mentioned for other SCBs, as well as JWH-018 (De Luca et al. 2015a), JWH-250 and JWH-073 (Ossato et al. 2016). Moreover, AKB48 depressed spontaneous locomotion in ND4 Swiss-Webster mice and positively substituted for the discriminative stimulus effects of Δ^9-THC in rats (Gatch and Foster, 2015). Therefore, the present study was aimed at investigating the acute effect of AKB48 and 5F-AKB48 (0.01-6 mg/kg i.p.) on body temperature, acute mechanical and thermal analgesia, catalepsy, motor activity, sensorimotor responses (to visual, acoustic and tactile stimulation), neurological changes (convulsion, hyperreflexia, and myoclonia), aggressive response and modulation of DAergic release in mesoaccumbal pathway in CD-1 mice. In vitro binding studies on CD-1 murine and human CB_1/CB_2 receptors have been also performed. Moreover, to better understand the behavioral effects of the AKB48 and 5F-AKB48, their actions were compared with those of JWH-018 and Δ^9-THC and effects were monitored for over 5 h.

2. Materials and methods

2.1. Animals

Male ICR (CD-1®) mice, 25-30 gr (ENVIGO 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 AM to 6:30 PM), temperature of 20-22 °C, humidity of 45-55% and were provided ad libitum access to food (Diet 4RF25 GLP; Mucedola, Settimo Milanese, Milan, Italy) and water. Experimental protocols performed in the present study were in accordance with the European Communities Council Directive of September 2010 (2010/63/EU) and were approved by Italian Ministry of Health (license n. 335/2016-PR) and by the Ethics Committee of the University of Ferrara. Moreover, adequate measures were taken to minimize the number of animals used and their pain and discomfort.

2.2. Drug Preparation and dose selection

AKB48 and 5F-AKB48 were purchased from LGC Standards (LGC Standards S.r.l., Sesto San Giovanni, Milan, Italy) and www.chemicalservices.net, while AM 251 was purchased from Tocris (Tocris, Bristol, United Kingdom). Drugs were initially dissolved in absolute ethanol (final concentration was 2%) and Tween 80 (2%) and brought to the final volume with saline (0.9% NaCl). The solution made of ethanol, Tween 80 and saline was also used as the vehicle. The CB_1 receptor-preferring antagonist/inverse agonist AM 251 (6 mg/kg) was administered 20 minutes before AKB48 and 5F-AKB48 injections. Drugs were administered by intraperitoneal injection at a volume of 4ul/g. A novel set of Δ^9-THC and JWH-018 data (stimulated aggressiveness) has been done in the present study. While, doses of AKB48 and 5F-AKB48 (0.01-6 mg/kg i.p.) were chosen based on previous studies (Vigolo et al. 2015; Ossato et al. 2015; Ossato et al. 2016).

2.3. Mouse tissues and cell culture membrane preparation

After mice were sacrificed by cervical dislocation, brain and spleen were removed and suspended in 50 mM Tris HCl buffer, pH 7.4 at 4°C. The mouse brain suspension was homogenized with a Polytron and centrifuged for 20 min at 40,000 x g. The mouse spleen was homogenized with a Polytron and centrifuged for 10 min at 2,000 x g. The supernatant was filtered and centrifuged for 20 min at 40,000 x g. The resulting pellets were used for competition binding experiments (Vincenzi et al. 2013). CHO cells transfected with human CB_1 or CB_2 receptors (Perkin Elmer Life and Analytical Sciences, USA) were grown adherently and maintained in Ham's F12 containing 10 % fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and Geneticin (G418, 0.4 mg/ml) at 37°C in 5 % CO_2/95 % air. For membrane preparation the culture medium was removed

and the cells were washed with PBS and scraped off plates in ice-cold hypotonic buffer (5 mM Tris HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized with a Polytron and then centrifuged for 30 min at 40,000 x g. The membrane pellet was suspended in 50 mM Tris HCl buffer (pH 7.4) containing 2.5 mM EDTA, 5 mM $MgCl_2$, 0.5 mg/ml BSA for CB_1 receptors or in 50 mM Tris HCl (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$, 0.5 mg/ml BSA for CB_2 adenosine receptors (Vincenzi et al. 2013).

2.4. [^{3}H] CP-55,940 competition binding assays and cyclic AMP assays

Competition binding experiments were performed as previously reported (Vincenzi et al. 2013; Vigolo et al., 2015) using 0.5 nM [^{3}H]-CP-55,940 and different concentrations of the tested compounds with membranes obtained from CHO cells transfected with human CB_1 or CB_2 receptors (2 µg protein/100 µl). Competition binding experiments were also performed in mouse brain membranes (40 µg protein/100 µl) for CB_1 receptors and in mouse spleen membranes (80 µg protein/100 µl) for CB_2 receptors. Non-specific binding was determined in the presence of 1 µM WIN 55,212-2. The filter bound radioactivity was counted using a Packard Tri Carb 2810 TR scintillation counter. Cyclic AMP assays were carried out in CHO cells transfected with human CB_1 or CB_2 receptors which were washed with PBS, detached with trypsin and centrifuged for 10 min at 200 x g (Vincenzi et al. 2013; Vigolo et al. 2015). The pellet was suspended in 0.5 ml of incubation mixture: 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM Hepes, 10 mM $MgCl_2$, 5 mM glucose, pH 7.4 at 37°C. Then, 0.5 mM Ro 20-1724 as a phosphodiesterase inhibitor was added and pre-incubated for 10 min in a shaking bath at 37°C. The potency of the examined compounds was studied in the presence of forskolin 1 µM. The reaction was terminated by the addition of cold 6% trichloroacetic acid and the final aqueous solution was tested for cyclic AMP levels by a competition protein binding assay.

2.5. Behavioural studies

The effect of AKB48 and 5F-AKB48 was investigated using a battery of behavioral tests widely used in studies of "safety-pharmacology" for the preclinical characterization of new molecules in rodents (Irwin, 1968; Mattsson et al. 1996; Porsolt et al. 2002; Redfern et al. 2005; Hamdam et al. 2013; S7A 2001). Those tests have been also validated to describe effects of cannabinoids on the “tetrad”, sensorimotor and neurological changes in mice (Compton et al. 1992; Vigolo et al. 2015; Ossato et al. 2015; Ossato et al. 2016). To reduce the number of animals used, the behaviour of mice was evaluated in five consecutive experimental sections (for detailed information see Supplementary Material). Moreover, to reduce the animal’s stress induced by manipulation, and to

confirm the stability and reproducibility over time of the responses of our tests, animals were trained 2 times per week for 2 weeks before the pharmacological treatment. All experiments were performed between 8:30 AM to 2:00 PM. Experiments were conducted in blind by trained observers working together in pairs (Redfern et al. 2005). The behavior of mice (neurologic and sensorimotor responses) was videotaped and analyzed off-line by a different trained operator that gives test scores.

2.5.1. Major neurological changes and aggressive response

As previously described by others studies (Vigolo et al. 2015; Ossato et al. 2015; Ossato et al. 2016), tail elevation, hyperreflexia, myoclonus, convulsions and aggressive responses in mice were observed immediately after SCBs administration (for detailed information see Supplementary Material). The tail elevation was measured during the observation of the freely moving mice in a square area (score 0/4 not tail elevation, score 4/4 Straub tail). Spontaneous aggressive response was measured based on the number of bites that the freely moving mouse confers to an object of gray cloth that approaches the front of the snout of the animal. While in the case of stimulated aggressiveness the animal is manually restrained and it is held in a supine position following which an object is brought near its mouth. For both spontaneous and stimulated aggressive behavior tests a gray cloth was placed in front of the mouse's nose for 10 consecutive times (score 0/10 not aggressive, score 10/10 very aggressive).

2.5.2. Sensorimotor studies

We studied the voluntary and involuntary sensorimotor responses resulting from different mouse reaction to visual, acoustic and tactile stimuli (Koch 1999; Marti et al. 2013; Ossato et al. 2015; for detailed information see Supplementary Material).

Visual response was verified by two behavioural tests, which evaluated the ability of the mouse to capture visual information even when the animal is moving (the visual placing response) or when it is stationary (the visual object response). *Visual Placing response* test is performed using a tail suspension modified apparatus able to bring down the mouse towards the floor at a constant speed of 10 cm/sec (Ossato et al. 2015). *Visual object response* test was used to evaluate the ability of the mouse to see an object approaching from the front or the side, than inducing the animal to shift or turn the head or retreat it (Ossato et al. 2015).

Acoustic response measures the reflex of the mouse in replay to an acoustic stimulus produced behind the animal (Koch 1999).

The tactile response in the mouse was verified through vibrissae, pinna and corneal reflexes (Ossato et al. 2015).

2.5.3. "Tetrad" paradigm for screening cannabinoid-like effect

To better assess the effects of the ligands on thermoregulation, we measured both changes in the core (rectal) and surface (ventral fur) temperature (for detailed information see Supplementary Material). The *core temperature* was evaluated by a probe (1 mm diameter) that was gently inserted, after lubrication with liquid vaseline, into the rectum of the mouse (to about 2 cm) and left in position until the stabilization of the temperature (about 10 sec; Vigolo et al. 2015). The probe was connected to a Cole Parmer digital thermometer (model 8402). The *surface temperature* was measured by a Microlife FR 1DZ1 digital infrared thermometer, placed at 1 cm from the surface of the abdomen of the mouse (Vigolo et al. 2015).

Acute mechanical and thermal nociception was evaluated respectively using the tail pinch and the tail withdrawal test (Vigolo et al. 2015; for detailed information see Supplementary Material).

Alterations of motor activity induced by AKB48 and 5F-AKB48 were measured using the bar, drag, accelerod tests and the analysis of spontaneous locomotor activity (Marti et al. 2004; Marti et al. 2005; Vigolo et al. 2015; Ossato et al. 2015; for detailed information see Supplementary Material).

2.5.4. In vivo brain microdialysis studies

Male ICR (CD-1®) mice, 25-30 g (ENVIGO. Harlan Italy; S. Pietro al Natisone, Italy) were anaesthetized with Sodium Penthobarbital (50 mg/kg i.p.; Sigma-Aldrich, Italy) and implanted with vertical dialysis probe (1 mm dialyzing portion) prepared with AN69 fibers (Hospal Dasco, Bologna, Italy) in the Nucleus Accumbens shell (NAc shell; A+1.4, L 0.4 from bregma, V-4.8 from dura) according to the mouse brain atlas by Paxinos and Franklin (Second Edition, 2001). On the day following surgery, probes were perfused with Ringer's solution (147 mM NaCl, 4 mM KCl, 2.2 mM $CaCl_2$) at a constant rate of 1 µl/min. Dialysate samples (15 µl) were injected into an HPLC equipped with a reverse phase column (C8 3.5 um, Waters, USA) and a coulometric detector (ESA, Coulochem II) to quantify DA. The first electrode of the detector was set at +130 mV (oxidation) and the second at -175 mV (reduction). The composition of the mobile phase was: 50 mM NaH_2PO_4, 0.1 mM Na_2-EDTA, 0.5 mM n-octyl sodium sulfate, 15 % (v/v) methanol, pH 5.5. The sensitivity of the assay for dopamine (DA) was 5 fmol/sample. At the end of each experiment, animals were sacrificed and their brains removed and stored in formalin (8 %) for histological examination to verify the correct placement of the microdialysis probe.

2.6. Data and statistical analysis

Protein concentrations were determined according to a Bio-Rad method with bovine serum albumin as reference standard. Inhibitory binding constants (Ki) were calculated from the IC_{50} values according to the Cheng and Prusoff equation: $Ki = IC_{50}/(1 + [C^*]/K_D^*)$, where [C*] is the concentration of the radioligand and K_D^* its dissociation constant. Functional experiments were analyzed by non-linear regression analysis using the equation for a sigmoid concentration-response curve using Prism (GraphPad Prism, USA). All data are expressed as the mean ± SEM of 3 independent experiments. Core and surface temperature values are expressed as the difference between control temperature (before injection) and temperature following drug administration (Δ°C). Antinociception (tail withdrawal and tail pinch tests) and catalepsy (bar test) are calculated as percent of maximal possible effect {EMax%=[(test - control latency)/(cut off time - control)] X 100}. Data are expressed in absolute values (sec in neurological changes and immobility time, m for distance travelled, m/sec for calculation of maximum speed and n° of bites in the aggressive response test), Δ°C (core and surface temperature), Emax% (tail withdrawal, tail pinch and bar test) and percentage of basal (drag test and accelerod test). In sensorimotor response experiments data are expressed in arbitrary units (visual objects response, acoustic response, vibrissae, corneal and pinna reflex) and percentage of baseline (visual placing response). In microdialysis experiments data are expressed as percentage of DA basal values. All the numerical data are given as mean ± SEM. Data were analyzed by utilizing repeated measures ANOVA. Results from treatments showing significant overall changes were subjected to *post hoc* Tukey tests with significance for $p < 0.05$. The statistical analysis of the effects of the individual substances in different concentrations over time and that of antagonism studies in histograms were performed by two-way ANOVA followed by Bonferroni's test for multiple comparisons. The analysis of the total average effect induced by treatments (expressed in the panels D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. The Student's t-test was used to determine statistical significance ($P<0.05$) between two groups (see neurological changes). The statistical analysis was performed with the program Prism software (GraphPad Prism, USA). The detailed results of the statistical tests are detailed in the Supplementary Material.

3. Results

3.1. Affinity and potency of AKB48 and 5F-AKB48 for CB_1 and CB_2 cannabinoid receptors

Competition binding experiments performed in CHO cell membranes transfected with human CB_1 or CB_2 (Fig 1 panel A and B) receptors revealed affinity values of the examined compounds in the nanomolar range. The introduction of a fluorine group in the structure of AKB48 determined a slightly increase in the affinity for both CB_1 and CB_2 receptor subtypes. A similar ratio between the Ki value to human CB_2 and the Ki value to human CB_1 for AKB48 and 5F-AKB48 was observed, with values of 0.52 and 0.45, respectively (Table 1). Also in this case, competition binding experiments performed in mouse brain membranes (for CB_1 receptors Fig 1 C) and in mouse spleen membranes (for CB_2 receptors Fig 1 D) showed a better affinity for the fluorinated version of AKB48 for both the receptors (Table 1).

Cyclic AMP experiments were performed to evaluate the potency of the two compounds in CHO cells transfected with human CB_1 or CB_2 (Fig 1 panel E and F) receptors. Potency values were in accordance with affinity data obtained in competition binding experiments (Table 1). AKB-48 and 5F-AKB-48 behaved as full agonists as demonstrated by the capability to completely inhibit the forskolin-stimulated cAMP production (Fig 1, panel E and F).

3.2. Major neurological changes

Systemic administration of AKB48 and 5F-AKB48 (0.01-6 mg/kg i.p.) caused important neurological changes in mice (Table 2), while in vehicle-treated mice no neurological alterations were observed. In particular, administration of high doses (3 and 6 mg/kg, i.p.) of adamantyl compounds induced spontaneous convulsions, hyperreflexia and myoclonias in mice: those effects were not observed after the administration of Δ^9-THC (Table 2). AKB48 administered at 6 mg/kg induced convulsions in 10% of treated animals, while 5F-AKB48 administered at 3 and 6 mg/kg induced convulsions in 30% and 90% of treated animals respectively. AKB48 at 6 mg/kg induced seizures with latency and duration similar to those produced by JWH-018, while 5F-AKB48 at 6 mg/kg induced seizures with longer duration but same latency as those produced by AKB48 (Table 2).

AKB48 administered at 6 mg/kg induced hyperreflexia in 25% of treated animals, while 5F-AKB48 at 3 and 6 mg/kg induced hyperreflexia in 30% and 75% of mice, respectively (Table 2). AKB48 at 6 mg/kg induced hyperreflexia with same latency and quite longer duration than that produced by 5F-AKB48 and JWH-018.

AKB48 administered at 6 mg/kg induced myoclonias in 45% of treated mice while 5F-AKB48 at 3 and 6 mg/kg induced myoclonias in 90% and 100% of treated animals (Table 2). 5F-AKB48 at 6 mg/kg induced myoclonias with longer latency and duration than those produced by AKB48 and JWH-018.

AKB48, 5F-AKB48 and JWH-018 induced tail elevation in mice with comparable frequency, latency and duration at the dose of 3 mg/kg (Table 2). However, 5F-AKB48 at 3 mg/kg greater increased the degree of tail elevation than JWH-018 and AKB48.

Finally, AKB48 (3 and 6 mg/kg), 5F-AKB48 (1, 3 and 6 mg/kg) and JWH-018 (3 and 6 mg/kg) induced spontaneous and stimulated aggressiveness in mice. JWH-018, AKB48 and 5F-AKB48 (6 mg/kg) caused spontaneous aggressiveness in 90%, 50% and 100% of treated animals respectively. While, JWH-018, AKB48 and 5F-AKB48 (6 mg/kg) induced aggressive behaviour in 100%, 70% and 100% of treated mice respectively. 5F-AKB48 at 6 mg/kg induced a stimulated aggressiveness with comparable duration than JWH-018 but higher than AKB48 at the same dose. 5F-AKB48 at 1 and 3 mg/kg stimulated aggressive behaviour in 30% and 70% of treated mice respectively, and the effect induced at 3 mg/kg was greater respect to that caused by AKB48 at the same.

All neurological changes were prevented by the pre-treatment with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p. injected 20 min before AKB48, 5F-AKB48 and JWH-018 administration; data not shown).

3.3. Evaluation of the visual object response

Visual object response tended to stay the same in vehicle-treated mice over 5 hours observation (Fig 2 panel A and B) and the effect was similar to that observed in naïve untreated animals (data not shown). Systemic administration of AKB48 (0.01-6 mg/kg i.p.) dose-dependently reduced the visual object response in mice at all doses tested and the effect persisted up to 240 minutes after injection (Fig 2 panel A). Systemic administration of 5F-AKB48 (0.01-6 mg/kg i.p.) dose-dependently reduced the visual object response in mice at all doses tested and the effect persisted up to 5 hours of observation only for the highest doses of substance 3 and 6 mg/kg i.p. (Fig 2 panel B). The inhibition of visual object response induced by the highest dose of AKB-48 (6 mg/kg i.p.) and 5F-AKB48 (3 mg/kg i.p.) was prevented by the pre-treatment with AM 251 (6 mg/kg i.p., Fig 2 panel C) which alone did not alter the visual object response in mice. AKB48 and 5F-AKB48, inhibited the visual placing response in a prolonged manner although the effect appeared to be lower with respect to that induced by JWH-018 and Δ^9-THC at the same doses, while for doses of 3 and 6 mg/kg effects were similar to those induced by Δ^9-THC (Fig 2 panel D).

3.4. Evaluation of the acoustic response

Acoustic response tended to stay the same in vehicle-treated mice over 5 hours observation (Fig 3 panel A and B) and the effect was similar to that observed in naïve untreated animals (data not shown). Systemic administration of AKB48 transiently reduced the acoustic response at 3 mg/kg and 6 mg/kg doses tested up to 195 minutes after injection (Fig 3 panel A). Differently, 5F-AKB48 inhibited the acoustic response in a prolonged manner and the effect appeared to be higher than that induced by AKB48 at the same doses where the inhibitory effect persisted up to 5 hours (fig 3 panel B). The inhibition of acoustic response induced by AKB48 (6 mg/kg i.p.) and 5F-AKB48 (3 mg/kg i.p.) was prevented by the pre-treatment with AM 251 (6 mg/kg i.p., Fig 3 panel C) which alone did not alter the acoustic response in mice (data not shown). AKB48 and 5F-AKB48, inhibited the acoustic response in a prolonged manner at the highest dose tested. Although, while the effect of AKB48 appeared to be lower than that induced by JWH-018 and Δ^9-THC at the same doses, effects of 5F-AKB48 were similar to those evocated by JWH-018 (Fig 3 panel D).

3.5. Evaluation of the pinnae reflex

Pinnae reflex did not change in vehicle-treated mice over 5 hours observation (Fig 4 panel A and B) and the response was similar to that observed in naïve untreated animals (data not shown). Systemic administration of AKB48 did not alter the pinnae reflex in mice (Fig 4 panel A), contrarily to that presented after administration of 5F-AKB48 in which the effect was prolonged for the highest dose tested (3 and 6 mg/kg i.p.; Fig 4 panel B). The inhibition of pinnae reflex induced by 5F-AKB48 (3 mg/kg i.p.) was prevented by the pretreatment with AM 251 (6 mg/kg i.p., Fig 4 panel C) which alone did not alter the pinnae reflex in mice (data not shown). 5F-AKB48 dose-dependently reduced the pinnae reflex at 6 mg/kg and the effect appeared to be higher than that induced by JWH-018 and Δ^9-THC at the same doses (Fig 4 panel D).

3.6. Evaluation of the vibrissae reflex

Vibrissae reflex did not change in vehicle-treated mice over 5 hours observation (Fig 5 panel A and B) and the response was similar to that observed in naïve untreated animals (data not shown). Systemic administration of AKB48 did not alter the vibrissae reflex (Fig 5 panel A). Contrarily, the effects of 5F-AKB48 were evident after 15 min with the highest dose (6 mg/kg i.p.; Fig 5 panel B). The inhibition of vibrissae reflex induced by 5F-AKB48 (3 mg/kg i.p.) was prevented by the pre-treatment with AM 251 (Fig 5 panel C) which alone did not alter the vibrissae reflex in mice (data not shown). 5F-AKB48 impaired the vibrissae reflex at 3 and 6 mg/kg and the effect appeared to be similar to that induced by JWH-018 and higher than that induced by AKB48 and Δ^9-THC (Fig 5 panel D).

3.7. Evaluation of the corneal reflex

Corneal reflex did not change in vehicle-treated mice over 5 hours observation (Fig 6 panel A and B) and the response was similar to that observed in naïve untreated animals (data not shown). Systemic administration of AKB48 inhibited transiently at 3 and 6 mg/kg the corneal reflex in mice (Fig 6 panel A). On the other hand, 5F-AKB48 inhibited deeply the corneal reflex in mice at 3 and 6 mg/kg (Fig 6 panel B). The inhibition of corneal reflex induced by AKB48 (6 mg/kg i.p.) and 5F-AKB48 (3 mg/kg i.p.) was prevented by the pre-treatment with AM 251 (6 mg/kg i.p. Fig 6 panel C) which alone did not alter the corneal reflex in mice (data not shown). As previously reported the effects of 5F-AKB48 were higher than those induced by AKB48 at higher doses (3 and 6 mg/kg). Finally, the effects of 5F-AKB48 at 3 and 6 mg/kg i.p. seem to be more similar than those induced by JWH-018 but higher than those induced by Δ^9-THC and AKB48 at same doses (Fig 6 panel D).

3.8. Evaluation of the visual placing response

Visual placing response tended to be reduced in vehicle-treated mice over 5 hours observation (~47% of reduction at 300 min; Fig 7 panel A and B) and the effect was similar to that observed in naïve untreated animals (data not shown). Systemic administration of AKB48 transiently reduced the visual placing response in mice at 3 and 6 mg/kg i.p. and the effect persisted up to 130 minutes (Fig 7 panel A). Systemic administration of 5F-AKB48 reduced the visual placing response in mice at all doses tested (0.01-6 mg/kg i.p.) and the effect persisted up to 5 hours only for the highest dose considered (6 mg/kg i.p. Fig 7 panel B). The visual impairment induced by AKB48 and 5F-AKB48 was prevented by the pre-treatment with AM 251 (6 mg/kg i.p., Fig 7 Panel C) which alone did not alter the parameter. The inhibition of the visual response induced by 5F-AKB48 are more higher than those induced by AKB48 and similar to those induced by Δ^9-THC (Fig 7 panel D).

3.9. Bar test

AKB48 and 5F-AKB48 induced catalepsy in the bar test (Fig 8 panel A e B). In particular, AKB48 induced a transient increase in the time spent on bar at 3 mg/kg and a marked catalepsy at 6 mg/kg which gradually decreases to baseline levels after 95 min from administration of AKB48 (Fig 8 panel A). 5F-AKB48 induced a marked catalepsy at 3 and 6 mg/kg and the effects remained up to 270 minutes (Fig 8 panel B). The effects were prevented by the pretreatment with AM 251 which alone did not induce akinesia and catalepsy (Fig 8 panel C). The effects of 5F-AKB48 were more intense than those induced by JWH-018, AKB48 and Δ^9-THC at the same doses considered effective (Fig 8 panel D).

3.10. Evaluation of core and surface body temperature

Systemic administration of AKB48 and 5F-AKB48 (0.01-6 mg/kg ip) reduced both core (Fig 9) and surface (Fig 10) body temperatures in mice. In particular, AKB48 induced a transient reduction in core temperature at 3 mg/kg (-2°C at 85 min time point) and a prolonged and significant hypothermia at 6 mg/kg (-5.5 °C at 85 min time point; Fig 9 panel A) that was maintained up to 140 minutes. Moreover, 5F-AKB48 induced a prolonged and significant hypothermia at both 3 mg/kg and 6 mg/kg in mice (Fig 9 panel B). AKB48 and 5F-AKB48 were ineffective in the range of doses of 0.01-1 mg/kg. Internal body hypothermia was associated by a reduction of the external body temperature which was observed only at the higher dose tested (6 mg/kg for AKB48 and 3-6 mg/kg for 5F-AKB48; Fig 10 panel A and B). Core and surface temperature changes were prevented by the pre-treatment with AM 251 which did not affect body temperature when administered alone (Fig 9 and 10 panel C). Furthermore, the effects on core temperature of AKB48 and 5F-AKB48 seem to be similar to those induced by JWH-018, while Δ^9-THC was less effective (Fig 9 panel D). The effects on surface temperature of AKB48 seem to be less effective than those induced by JWH-018 and 5F-AKB48 (Fig 10 panel D).

3.11. Evaluation of pain induced by a mechanical stimulus

Systemic administration of AKB48 and 5F-AKB48 (0.01-6 mg/kg i.p.) increased the threshold to acute mechanical pain stimulus in mice in the tail pinch test (Fig 11). In the case of AKB48, only the dose of 6 mg/kg was transiently effective from 55 to 90 minutes of analysis (Fig 11 panel A). On the other hand, 5F-AKB48 was active in the all dose range of 0.01-6 mg/kg (Fig 11 panel B) and the effects were prolonged up to 5 hours after injection of the compound. The effects were prevented by the pre-treatment with AM 251 which alone did not alter the threshold to acute mechanical pain stimuli (Fig 11 panel C). It is interesting to note that for 5F-AKB48 the anti-nociceptive effect was already significant at the lower dose tested (0.01 mg/kg, panel B) and it induced an increase in the pain threshold more higher than that induced by the same doses (3-6 mg/kg i.p.) of AKB48 (Fig 11 panel D).

3.12. Evaluation of pain induced by a thermal stimulus

Systemic administration of AKB48 and 5F-AKB48 (0.01-6 mg/kg i.p.) increased the threshold to acute thermal pain stimulus in mice in the tail withdrawal test (Fig 12 panel A and B). In particular, AKB48 induced a robust elevation of the pain threshold at 6 mg/kg which ended after 145 min after administration of the compound (Fig 12 panel A). Also 5F-AKB48 induced a robust elevation of the pain threshold at 6 mg/kg but the effect persisted up to 5 hours (Fig 12 panel C). The effects

were prevented by the pretreatment with AM 251 which alone did not alter the threshold to acute thermal pain stimuli (Fig 12 panel C). At 6 mg/kg, AKB48 and 5F-AKB48 induced an increase in the pain threshold similar to that induced by the same dose of Δ^9-THC (Fig 12 panel D).

3.13. Accelerod test

In the accelerod test, AKB48 transiently inhibited stimulated locomotion only at 6 mg/kg (Fig 13 panel A). Conversely, 5F-AKB48 induced at the highest dose tested (3 and 6 mg/kg i.p.) a prolonged and significant impairment of locomotion (Fig 13 panel B). The inhibitory effects were prevented by the pre-treatment with the AM 251, which alone did not affect mice performance (Fig 13 panel C). AKB48 and 5F-AKB48 caused an effect lower than that induced by JWH-018 at 6 mg/kg (Fig 13 panel D).

3.14. Drag test

Systemic administration of AKB48 transiently inhibited the number of steps performed with the front legs of the mice at 3 mg/kg and 6 mg/kg (Fig 14 panel A). On the other hand, the effects of 5F-AKB48 were evident also with the dose of 1 mg/kg (Fig 14 panel B). At 6 mg/kg the effect of 5F-AKB48 was prolonged and persisted up to 5 hours observation. The inhibitory effects were prevented by the pretreatment with the AM 251 (Fig 14 panel C). The inhibition induced by 5F-AKB48 was similar to those induced by JWH-018 and greater respect to those caused by AKB48 and Δ^9-THC at the same doses (Fig 14 panel D).

3.15. Studies on spontaneous locomotor activity in mice

To exclude that the reduction of sensorimotor responses could be due to the inhibition of motor activity, we investigated the effect of AKB48 and 5F AKB48 administration (0.01-6 mg/kg i.p.) on spontaneous locomotor activity in mice. AKB48 at 6 mg/kg reduced the total distance travelled (Fig 15 A) and increased the immobility time at 1 and 6 mg/kg (Fig 15 D) in mice. To be noted that AKB48 at 1 mg/kg evoked a transient facilitation of spontaneous locomotion 15 min after drug injection (Fig 15 A). Likewise, 5F-AKB48 at 6 mg/kg reduced the total distance travelled (Fig 15 B) and increased at 1 and 6 mg/kg the immobility time (Fig 15 E) in mice. 5F-AKB48 induced a greater inhibition of total distance travelled respect AKB48 administration (Fig 15 C) without changing the total immobility time (Fig 15 F).

3.16. In vivo brain microdialysis

Basal values of extracellular DA in NAc shell were 15 ± 5 (mean ± SEM) fmoles/10 µl sample. Systemic administration of AKB48 (0.3 and 1 mg/kg i.p.) and 5F-AKB48 (0.01, 0.03, 0.1 and 0.3 mg/kg/i.p.) increased extracellular DA release in NAc shell of awake and freely moving mice (Fig 16 panel A and B) in a dose-dependent manner. In particular, AKB48 increased DA levels in the NAc shell with a biphasic effect after the administration of the highest dose tested (1 mg/kg/ip); no effects were observed with the lowest dose as with vehicle. Moreover, the administration of the fluorinated analog, 5F-AKB48, produced a dose-response curve with the dose of 0.03 mg/kg increasing dialysate DA; no effects were observed with the lower and higher doses tested as with vehicle. 5F-AKB48 at 0.03 mg/kg induced a prolonged release of DA (up to 180 minutes) that reached the maximum at 30-60 min after drug administration (max increase of about +75 %) showing differences at 30, 40 and 60 min samples with respect to basal values.

4. Discussion

This is the first study showing a comparative analysis of the effects caused by the new third-generation synthetic cannabinoids AKB48 and 5F-AKB48 on "tetrad", sensorimotor, neurological and neurochemical responses in CD-1 male mice.

Firstly, the study shows that the systemic administration of AKB48 and 5F-AKB48 induces the typical “tetrad effect” as reported for other JWH-type SCBs (Wiebelhaus et al. 2012; Wiley et al. 1998; Macri et al. 2013; Vigolo et al. 2015; Ossato et al. 2016) and Δ^9-THC (Compton et al. 1992; Vigolo et al. 2015). In particular, effects induced by AKB48 on “tetrad” appear to be less potent than those induced by 5F-AKB48 and more comparable with that of Δ^9-THC. Conversely, 5F-AKB48 displays an overall activity on “tetrad” similar to that caused by JWH-018. Moreover, the study shows that AKB48 and 5F-AKB48 cause important alteration of sensorimotor reflexes and they promote spontaneous and stimulated aggressive response in mice.

Furthermore, as previously reported regarding the synthetic cannabinoids JWH-018, JWH-250 and JWH-073, these two adamantylindazoles induce neurological alterations such as convulsions, hyperreflexia and myoclonias that are not observed after administration of Δ^9-THC (Marshell et al. 2014; Vigolo et al. 2015; Ossato et al. 2016).

Finally, by the microdialysis technique in awake and freely moving mice, we demonstrated that systemic administration of AKB48 and 5F-AKB48 transiently facilitates extracellular DA release in the NAc shell. All these behavioural and neurochemical effects were fully dependent on CB_1 receptor stimulation since they are completely prevented by the administration of the selective CB_1 receptor antagonist/inverse agonist AM 251.

The protocol we used in this study was previously validated to describe the effects of other cannabinoids on the “tetrad”, sensorimotor and neurological changes in mice (Vigolo et al. 2015; Ossato et al. 2015; Ossato et al. 2016).

In vitro binding studies show that AKB48 and 5F-AKB48 retain nanomolar affinity for both CD-1 murine and human CB_1 and CB_2 receptors with a slightly preference for CB_2 receptor (Uchiyama et al., 2013). In particular, in CD-1 murine preparation AKB48 displays an affinity for CB_1 receptors (Ki = 5.34 nM) similar to that of 5F-AKB48 (Ki = 3.87 nM) and JWH-018 (Ki = 5.82 nM; (Vigolo et al. 2015). Whereas, on human CB_1 receptors, AKB48 shows an affinity (Ki = 3.24 nM) compared to that of 5F-AKB48 (Ki = 1.82 nM) but slightly higher to that JWH-018 (Ki = 9.53 nM; (Vigolo et al. 2015). The increased CB_1 receptor affinity of AKB48 and 5F-AKB48 could justify their potency value (AKB48, IC_{50} = 5.39 nM and 5F-AKB48, IC_{50} = 2.57 nM) in inhibiting cyclic AMP formation respect to JWH-018 (IC_{50} = 14.1 nM; (Vigolo et al. 2015). Despite these in vitro evidence show that AKB48 and 5F-AKB48 have an affinity for the CB_1 receptors equal or slightly greater

than JWH-018, in vivo data show a different efficacy and potency between AKB48, 5F-AKB48 and JWH-018. This is suggestive of the fact that the in vivo efficacy of these compounds does not depend exclusively on pharmacodynamic (i.e. receptor affinity) but possibly by pharmacokinetic (i.e. absorption, metabolism) parameters. This is supported by recent studies that have shown that the halogenation in the pentilic side chain of JWH-018 (i.e. JWH-018Cl and JWH-018Br) does not significantly change the binding affinity of the compounds at the cannabinoid CB_1 and CB_2 receptors, but it influences their biological activity in vivo (Vigolo et al. 2015). Therefore, the increased power of 5F-AKB48 compared to AKB48 could be due to the enhanced lipophilicity of the fluorinate compound (Schifano et al. 2015).

Indeed, administration of AKB48 in the dose-range up to 6 mg/kg induces a core and surface hypothermia which is significantly lower respect to that induced by 5F-AKB48 and JWH-018, and it was similar to that induced by administration of Δ^9-THC (Vigolo et al. 2015). Nevertheless, we cannot exclude that administration of AKB48 at higher doses than those tested might induce a greater hypothermia. However, the occurrence of major neurological changes prevents us to increase doses. As reported for others cannabinoid agonists, hypothermia induced by AKB48 and 5F-AKB48 is completely prevented by pretreatment with AM 251 confirming that this effect is clearly mediated by the stimulation of CB_1 receptors (Marshell et al. 2014; Vigolo et al. 2015; Ossato et al. 2016).

Systemic administration of AKB48 and 5F-AKB48 increases the threshold to acute mechanical and thermal pain stimulus in mice. However, the analgesic effect induced by AKB48 is less intense respect to that induced by 5F-AKB48, JWH-018 and Δ^9-THC administration (Vigolo et al. 2015) but it is similar to the analgesic profile of other SCBc as JWH-250 and JWH-073 (Ossato et al. 2016). This lower response could be due to the fact that AKB48, as well as others SCBs, may be biotransformed into glucuronitated or monohydroxylated metabolites that can act as neutral antagonists at CB_1 receptors dampening the overall activity of the parent compound (Seely et al. 2012; Brents et al. 2012). However, the structural similarity between AKB48 and 5F-AKB48 suggests that the lower efficacy of AKB48 is more likely related to its lower permeation across the blood brain barrier. As previously reported for others JWH-type SCBs (Vigolo et al. 2015), 5F-AKB48 shows a greater efficacy in reducing nociception to mechanical stimulation compared to thermal stimulus, strengthens the hypothesis that cannabinoid agonists exert their analgesic effect by acting on different sensory components of pain generated by a mechanical (Martin et al. 1996) or thermal (Hohmann et al. 1999) stimuli.

Unlike previous studies showing that the analgesic effect caused by JWH-018-R compounds precedes the motor impairment (Vigolo et al. 2015), the analgesic effects induced by AKB48 and

5F-AKB48 overlap almost completely to the motor alterations. This responsiveness is in line with previous studies reporting that small changes in the molecular structure of SCBs induce consistent disparities among potencies and efficacies of in vivo effects (Wiley et al. 1998; Wiley et al. 2014; Ossato et al. 2016).

In our experimental conditions, the possibility that the acute analgesic effect induced by AKB48 and 5F-AKB48 and/or their metabolites (Ghandi et al. 2013; Holm et al. 2015) is due to the activation of peripheral CB_2 receptors (Guindon and Hohmann, 2008) should be ruled out since their analgesic effects are fully prevented by the administration of the selective CB_1 receptor antagonist/inverse agonist AM 251.

Administration of AKB48 and 5F-AKB48 affects, less effectively than JWH-018 and Δ^9-THC, the startle response to visual, acoustic and tactile stimuli in mice (Ossato et al. 2015). A recent study has shown that visual information in mice is elaborated in a subpopulation of neurons selectively localized in the dorsomedial striatum (Reig and Silberberg 2014), in which CB_1 receptors are expressed (Tsou et al. 1998; Marsicano and Lutz 1999). Even though in our study we are not able to understand which brain areas and neural mechanisms are responsible for the reduced visual response of the mouse, it is possible to hypothesize that AKB48 and 5F-AKB48 could inhibit visual function through the stimulation of CB_1 receptors expressed in thalamocortical-striatal visual circuitry (Tsou et al. 1998; Marsicano and Lutz 1999; Dasilva et al. 2012; Yoneda et al. 2013). Our study also demonstrates that AKB48 and 5F-AKB48 impair the acoustic startle response in mice by the selective stimulation of CB_1 receptors. This finding is in agreement with previous researches that have demonstrated the effectiveness of acute administration of Δ^9-THC (Malone and Taylor 2006; Nagai et al. 2006; Ossato et al. 2015), CP 55940 (Mansbach et al. 1996; Martin et al. 2003), WIN-55,212-2 (Bortolato et al. 2005), JWH-018 (Ossato et al. 2015), JWH-250 and JWH-073 (Ossato et al. 2016) in reducing the acoustic startle reflex in rodents. Acoustic startle reflex is induced by the activation of three serially connected structures that involve the activation of the dorsal cochlear nucleus (Gomez-Nieto et al. 2014). Therefore, AKB48 and 5F-AKB48 could impair the acoustic startle reflex in mice by stimulating CB_1 receptors expressed on the presynaptic terminals of parallel fibers in the dorsal cochlear nucleus (Tzounopoulos et al. 2007).

Relying on the present study it is not possible to define whether visual and acoustic alterations induced by AKB48 and 5F-AKB48 in mice are an expression of hallucinatory states, as suggested for the Δ^9-THC in human studies (Winton-Brown et al. 2011). However, our data support the hypothesis that SCBs by stimulating CB_1 receptors could impair the sensorimotor gating in mice similarly to what demonstrated for other cannabinoid agonists such as Δ^9-THC (Malone and Taylor

2006; Nagai et al. 2006), CP 55940 (Mansbach et al. 1996; Martin et al. 2003) and WIN 55,212-2 (Schneider and Koch 2002; Wegener et al. 2008).

We also underline that 5F-AKB48 is more effective than AKB48 in inhibiting the sensorimotor responses in mice in reply to tactile stimuli. The inhibitory effect induced by 5F-AKB48 administration on vibrissae responses is consistent with previous studies showing that endocannabinoid system and exogenous Δ^9-THC or WIN-55,212-2 administration directly modulated whisking activity in rodent (Patel et al. 2002; Pietr et al. 2010; Ho et al. 2010) by activating CB_1 receptors (Tsou et al. 1998; Cristino et al. 2006) expressed in the inferior olive, somatosensory cortex and superior colliculus (Hemelt and Keller 2008). Similarly, 5F-AKB48 might inhibit sensorimotor responses of pinna and cornea through the stimulation of CB_1 receptors directly expressed in trigeminal structures (Herkenham et al. 1991; Tsou et al. 1998; Price et al. 2003) as hypothesized for JWH-018 (Ossato et al. 2015). These results are consistent with previous studies showing that the administration of HU 210 and WIN-55,212-2 suppressed central trigeminal transmission (Jenkins et al. 2004; Papanastassiou et al. 2004) and that topical application of WIN-55,212-2 reduced cornea-evoked trigeminal brainstem activity (Bereiter et al. 2002).

It is interesting to note that both AKB48 and 5F-AKB48 impair visual sensorimotor responses in mice at lower doses (0.1 and 1 mg/kg) that do not cause catalepsy or reduce spontaneous (open field studies) and stimulated motor activity (drag test and accelerod). These findings point out that effects induced by AKB48 and 5F-AKB48 on sensorimotor responses and motor activity are mediated by separate processes and suggest that the decreased sensory responsiveness does not result merely from a disruption of motor function (Ossato et al. 2015).

The present study showing that 5F-AKB48 is more potent in inducing convulsions respect to JWH-018 (Vigolo et al. 2015) probably due to its fluorination which determines a high lipophilicity and a quick pass across the blood-brain barrier (Schifano et al. 2015). These data confirm the proconvulsant effect of SCBs and they are in agreement with the increasing clinical reports showing the occurrence of seizures and hyperreflexia in young people who have smoked “Spice” products containing different SCBs (Gugelmann et al. 2014; Lapoint et al. 2011; McQuade et al. 2013; Schneir and Baumbacher 2012; Simmons et al. 2011).

As previously reported, SCBs promote aggressive response in mice (Ossato et al., 2016). Pharmacological modulation of cannabinoid signal alter spontaneous aggressive behaviour in mice, rats, and squirrel monkeys (Ham and De Jong 1975; Miczek 1978; van Ree et al. 1984) and this behaviour was exacerbate in stressful situations in rodents (Carder and Olson 1972; Carlini and Gonzales 1972; Carlini et al. 1976). Therefore, despite in our experiment this behaviour was observed in a simple test that is not fully representative for an overall and accurate assessment of

aggressive behavior in mice (Takahashi and Miczek 2014; Miczek et al. 2007), it is possible that the aggressive response caused by the administration of AKB48 and 5F-AKB48 in mice is mainly due to the stressful situation of the animal (sensorimotor alterations and neurological symptoms) rather than a direct effect on neural circuits that control aggressive behaviour.

In order to evaluate whether AKB48 and 5F-AKB48 share with natural and drug rewards the ability to increase DA transmission in the NAc shell, the effect of both drugs was evaluated by means of *in vivo* brain microdialysis in CD-1 mice. While the AKB48 increased dialysate DA levels in the NAc shell with a biphasic effect after the administration of the highest dose tested (1 mg/kg/i.p.), 5F-AKB48 displayed a bell-shaped dose-response curve with the dose of 0.03 mg/kg increasing DA. The present data confirm the rewarding properties of these third generation' SCBs and are similar to previous observation on the effect of JWH-018 (De Luca et al. 2015a) and BB-22 (De Luca et al. 2015b). Indeed, the specific increase of DA in the shell subdivision of the NAc is a common property of natural (Tanda et al. 1997) and synthetic cannabinoids (Fattore et al. 2005; Lecca et al. 2006), but also of drugs of abuse belonging to the most different pharmacological classes (Pontieri et al. 1995; Tanda et al. 1997; Di Chiara et al. 2004; Miliano et al. 2016). Importantly, this effect was observed at a very low doses compared to Δ^9-THC (Tanda et al. 1997) or to first generation SCBs such as WIN 55,212-2 (Fattore et al. 2005; Lecca et al. 2006) and JWH-018 (De Luca et al. 2015). The lack of increase of extracellular DA at hight doses might be due to the synthesis of hydroxylated metabolites of the SCBs, thus preventing the effect of the parent drug (Dhawan et al. 2006; Wiebelhaus et al. 2012). Another possible explanation might be a retrograde signaling through presynaptic CB_2 receptors located on DAergic terminals of the NAc (Xi et al. 2011; Morales and Bonci 2012). Interestingly, the inverted U-shaped dose-response curve with an extremely narrow range of doses appears to be peculiar to the SCBs.

5. Conclusion

For the first time the present study demonstrates the overall pharmacological effects induced by the administration of novel adamantylindazoles AKB48 and 5F-AKB48 in mice highlighting that the fluorination in the pentilic side chain of the indazolic structure increases the in vivo efficacy of SCBs, enhancing both the pharmacological activity and the adverse effects.

Table 1

Binding and functional parameters of AKB48 and 5F-AKB48 to human and mouse CB_1 and CB_2 receptors

Compound	*hCB1 CHO membranesa[a] Ki (nM)*	*hCB2 CHO membranes[a] Ki (nM)*	*Mouse cortex membranes CB1[a] Ki (nM)*	*Mouse spleen membranes CB2[a] Ki (nM)*	*hCB1 CHO cells[b] IC50 (nM)*	*hCB2 CHO cells[b] IC50 (nM)*
AKB48	3.24 ± 0.28	1.68 ± 0.12	5.34 ± 0.44	1.93 ± 0.14	5.39 ± 0.47	2.13 ± 0.21
5F-AKB48	1.82 ± 0.15	0.82 ± 0.07	3.87 ± 0.27	1.24 ± 0.07	2.57 ± 0.19	1.94 ± 0.14

Data are expressed as mean ± SEM.
[a] *[^{3}H]-CP-55,940 competition binding experiments.*
[b] *Cyclic AMP experiments.*

Table 2

Effects of the systemic administration of Δ^9-THC (0.01-100 mg/Kg i.p.), JWH-018, AKB48 and 5F-AKB48 (0.01-6 mg/Kg i.p.) on the neurological changes of the mouse.

Elevation tail

Compound	Vehicle	Δ^9-THC[a]							JWH-018[a]					AKB48					5F-AKB48				
Doses (mg/Kg)		0.01	0.1	1	3	6	30	100	0.01	0.1	1	3	6	0.01	0.1	1	3	6	0.01	0.1	1	3	6
Frequency (%)	–	–	–	–	–	–	10	65	–	–	10	35	80	–	–	–	40	70	–	–	–	30	75
Score	–	–	–	–	–	–	0.6±0.76	1.1±0.8	–	–	0.5±0.41	1.2±0.17	2.4±0.33	–	–	–	2.2±0.6	1.88±0.3	–	–	–	2.6±0.12 *	3.1±0.2 #
Duration (sec)	–	–	–	–	–	–	654.7±82.9	934.7±88.2	–	–	412.6±132.9	1236.8±111.5	1766.6±189.7	–	–	–	1007.7±146	998.5±89 **	–	–	–	1271±122	1436.1±157
Latency (sec)	–	–	–	–	–	–	112.5±33.9	103.6±17.4	–	–	92.5±17.1	94.8±16.3	88.6±13.4	–	–	–	99.7±24	101.2±21	–	–	–	88.2±18	91.2±10

Hyperrilexia

Compound	Vehicle	Δ^9-THC[a]							JWH-018[a]					AKB48					5F-AKB48				
Doses (mg/Kg)		0.01	0.1	1	3	6	30	100	0.01	0.1	1	3	6	0.01	0.1	1	3	6	0.01	0.1	1	3	6
Frequency (%)	–	–	–	–	–	–	–	–	–	–	–	15	80	–	–	–	–	25	–	–	–	30	75
Duration (sec)	–	–	–	–	–	–	–	–	–	–	–	1354.8±67.2	1439.8±45.3	–	–	–	–	1980.2±298	–	–	–	1009±221	1254.2±210
Latency (sec)	–	–	–	–	–	–	–	–	–	–	–	124.9±31.7	93.5±21.2	–	–	–	–	143±55	–	–	–	191.2±51	179±61

Myoclonie

Compound	Vehicle	Δ^9-THC[a]							JWH-018[a]					AKB48					5F-AKB48				
Doses (mg/Kg)		0.01	0.1	1	3	6	30	100	0.01	0.1	1	3	6	0.01	0.1	1	3	6	0.01	0.1	1	3	6
Frequency (%)	–	–	–	–	–	–	–	–	–	–	–	–	80	–	–	–	–	45	–	–	–	90	100
Duration (sec)	–	–	–	–	–	–	–	–	–	–	–	–	669.7±36.6	–	–	–	–	1087.6±241	–	–	–	1181.6±224	1593±299 *
Latency (sec)	–	–	–	–	–	–	–	–	–	–	–	–	109.7±16.35	–	–	–	–	185±19.7	–	–	–	224±41	210±36 *

Convulsion

Compound	Vehicle	Δ^9-THC[a]							JWH-018[a]					AKB48					5F-AKB48				
Doses (mg/Kg)		0.01	0.1	1	3	6	30	100	0.01	0.1	1	3	6	0.01	0.1	1	3	6	0.01	0.1	1	3	6
Frequency (%)	–	–	–	–	–	–	–	–	–	–	–	–	70	–	–	–	–	10	–	–	–	30	90
Duration (sec)	–	–	–	–	–	–	–	–	–	–	–	–	369.7±32.2	–	–	–	–	454.2±67.1	–	–	–	480.1±59	1821.3±457 ** ##
Latency (sec)	–	–	–	–	–	–	–	–	–	–	–	–	109.7±16.3	–	–	–	–	248±79	–	–	–	271.3±47.9	274.7±69.2

Spontaneous aggressiveness

Compound	Vehicle	Δ^9-THC[b]							JWH-018[b]					AKB48					5F-AKB48				
Doses (mg/Kg)		0.01	0.1	1	3	6	30	100	0.01	0.1	1	3	6	0.01	0.1	1	3	6	0.01	0.1	1	3	6
Frequency (%)	–	–	–	–	–	–	–	–	–	–	–	–	90	–	–	–	–	50	–	–	–	50	100
Score (n° of bites)	–	–	–	–	–	–	–	–	–	–	–	–	8.2±3.1	–	–	–	–	1.60±1.7	–	–	–	1.4±0.31	1.7±2.9
Duration (sec)	–	–	–	–	–	–	–	–	–	–	–	–	2750±621	–	–	–	–	2971.1±581	–	–	–	3303±512	3272.6±602
Latency (sec)	–	–	–	–	–	–	–	–	–	–	–	–	324.5±76	–	–	–	–	318.2±88	–	–	–	296.2±66	302.2±64

Stimulated aggressiveness

Compound	Vehicle	Δ^9-THC							JWH-018					AKB48					5F-AKB48				
Doses (mg/Kg)		0.01	0.1	1	3	6	30	100	0.01	0.1	1	3	6	0.01	0.1	1	3	6	0.01	0.1	1	3	6
Frequency (%)	–	–	–	–	–	–	–	–	–	–	–	50	100	–	–	–	35	70	–	–	30	70	100
Score (n° of bites)	–	–	–	–	–	–	–	–	–	–	–	5.1±4.3	10±0.2	–	–	–	1.75±1.7	4.37±3.9	–	–	3.75±4.1	7.3±3.6	4.75±2.9
Duration (sec)	–	–	–	–	–	–	–	–	–	–	–	12316±420	15286±408	–	–	–	3499±553	7217.1±677 ***	–	–	3051.8±590	14071±387 ###	14482±428 ###

Data are expressed as percentage (frequency of animal with neurological signs), seconds (duration and latency of neurological signs) and score (number of bites connected to spontaneous and stimulated aggressiveness and degree of elevation connected to the elevation tail), represent the mean ± SEM of 10 animals for each treatment. Statistical analysis was performed with one-way ANOVA followed by Tukey's test for multiple comparisons and Student's t-test was used to determine statistical significance ($P<0.05$) between two groups. *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus JWH-018 at the same dosage and #$p<0.05$, ##$p<0.01$, ###$p<0.001$ versus AKB48 at the same dosage.

[a] *from Vigolo et al. 2015*

[b] *from Ossato et al. 2016*

Figures

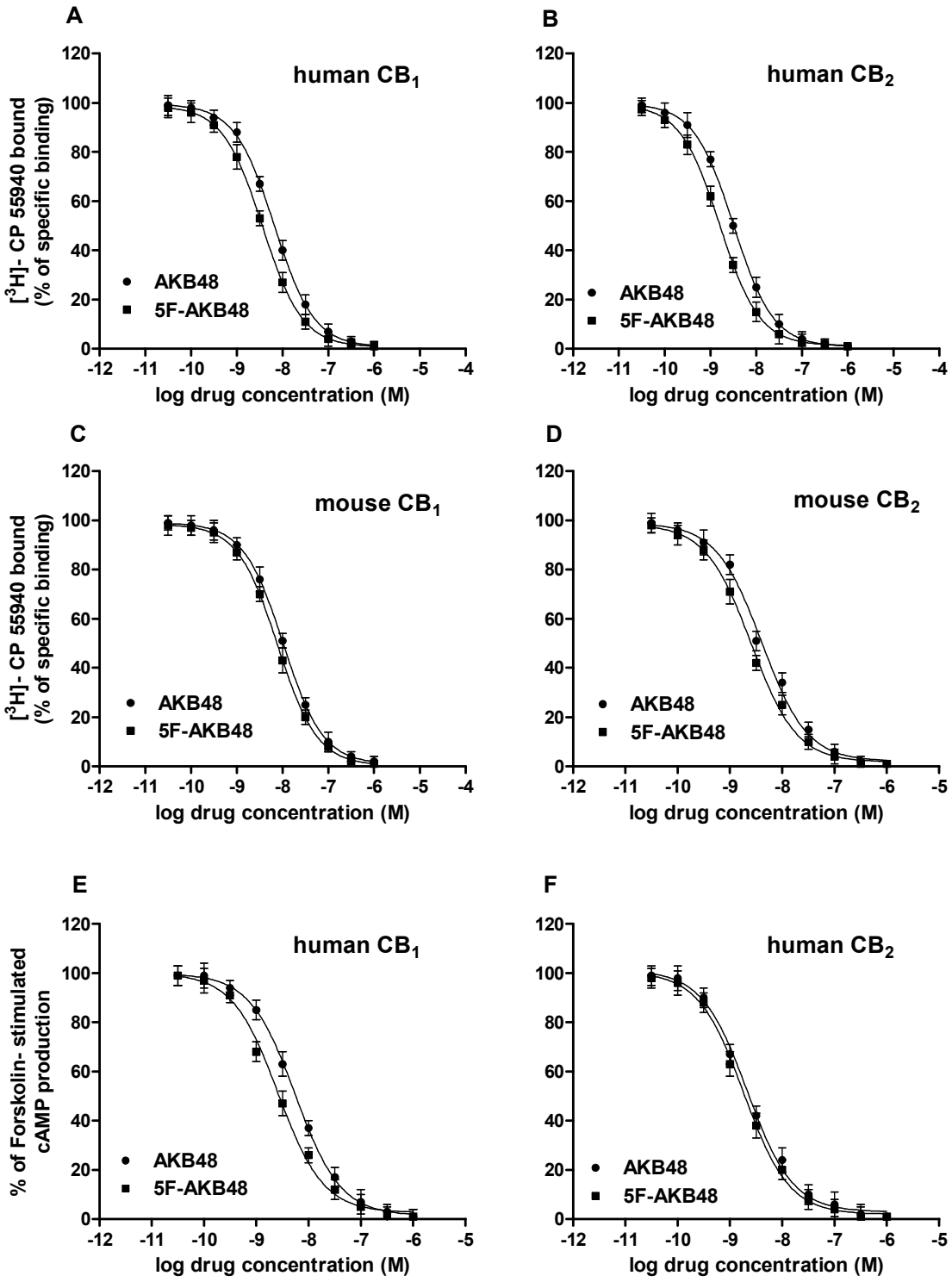

Figure 1

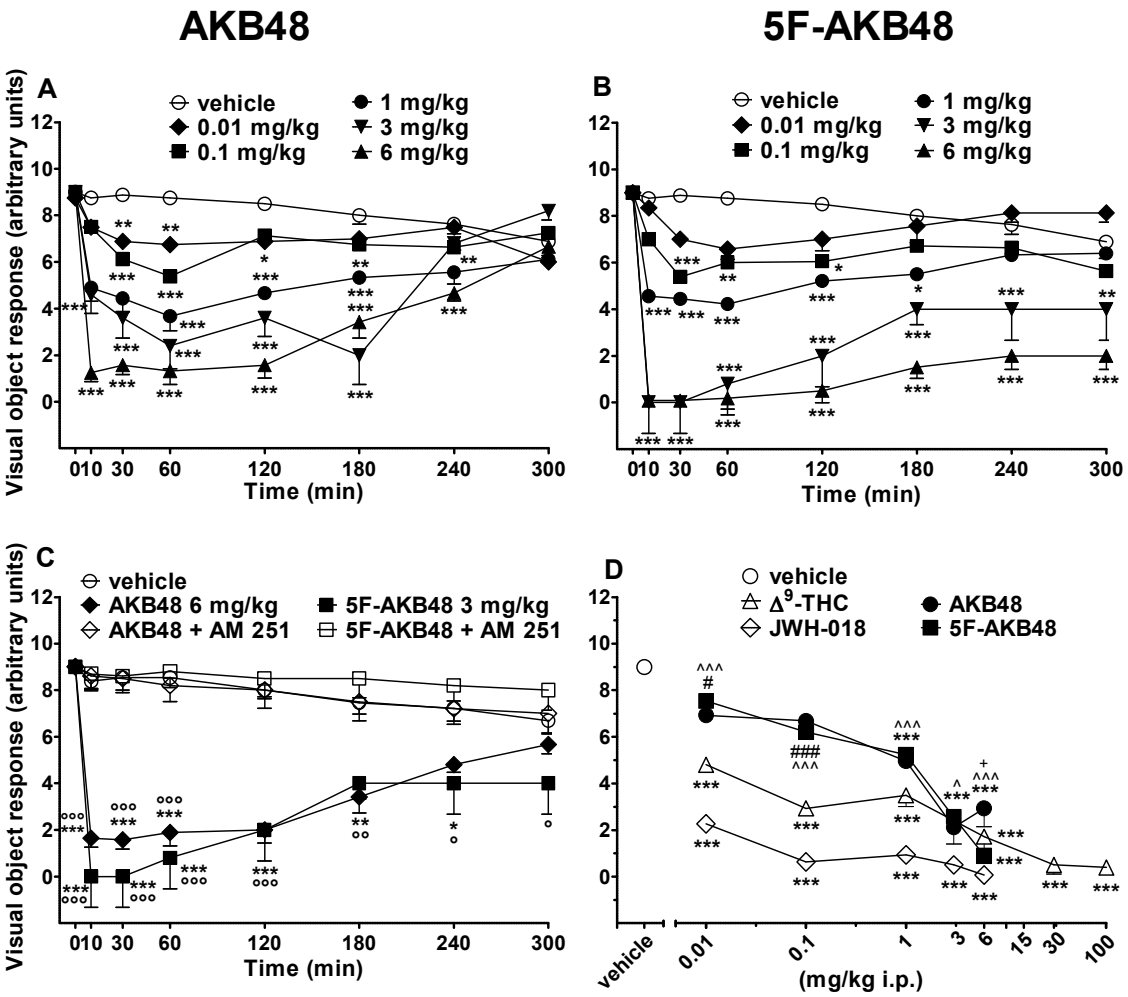
AKB48
5F-AKB48
A
B
C
D
vehicle
0.01 mg/kg
0.1 mg/kg
1 mg/kg
3 mg/kg
6 mg/kg
AKB48 6 mg/kg
5F-AKB48 3 mg/kg
AKB48 + AM 251
5F-AKB48 + AM 251
Δ9-THC
JWH-018
AKB48
5F-AKB48
Visual object response (arbitrary units)
Time (min)
(mg/kg i.p.)

Figure 2

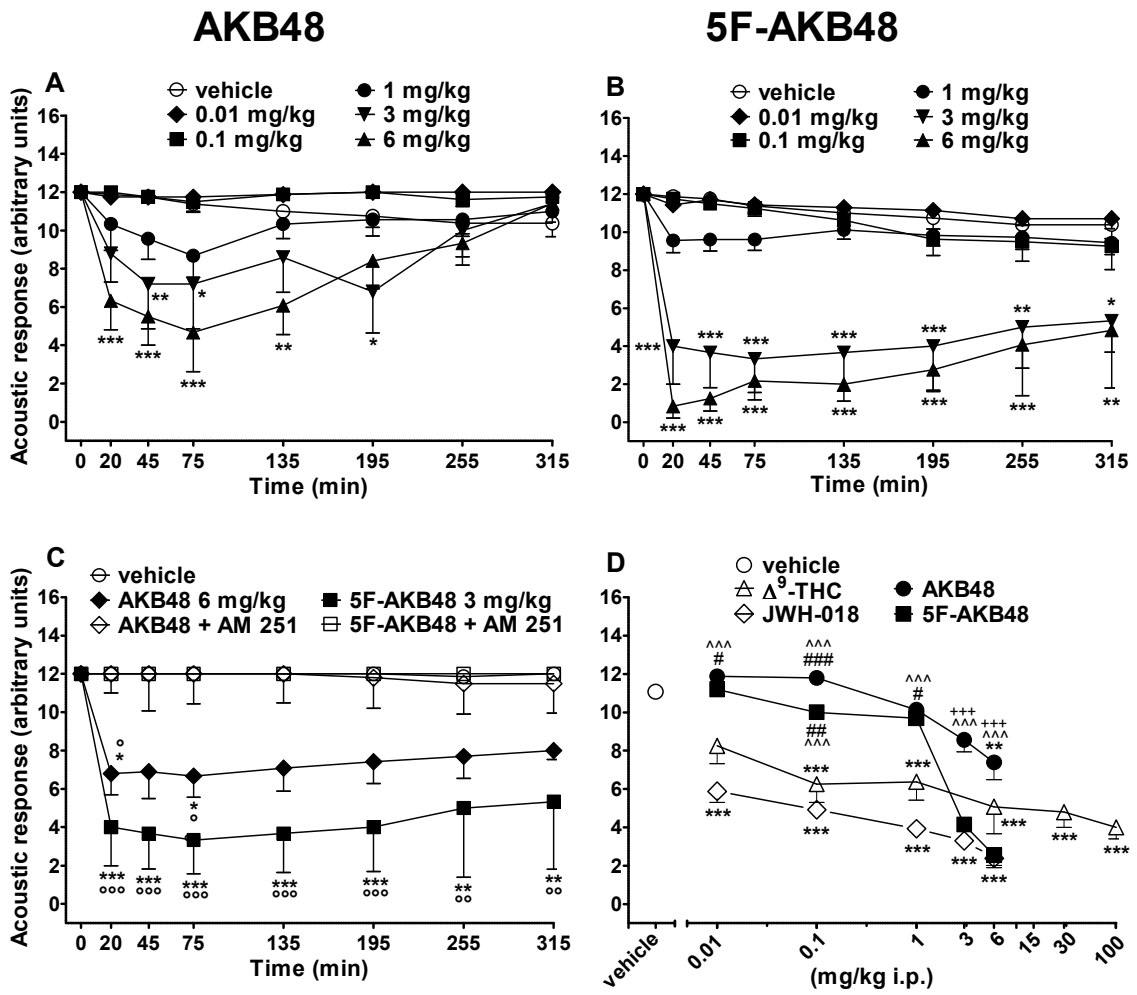
AKB48
5F-AKB48
A
B
C
D
vehicle
1 mg/kg
0.01 mg/kg
3 mg/kg
0.1 mg/kg
6 mg/kg
Acoustic response (arbitrary units)
Time (min)
AKB48 6 mg/kg
5F-AKB48 3 mg/kg
AKB48 + AM 251
5F-AKB48 + AM 251
Δ9-THC
JWH-018
AKB48
5F-AKB48
(mg/kg i.p.)

Figure 3

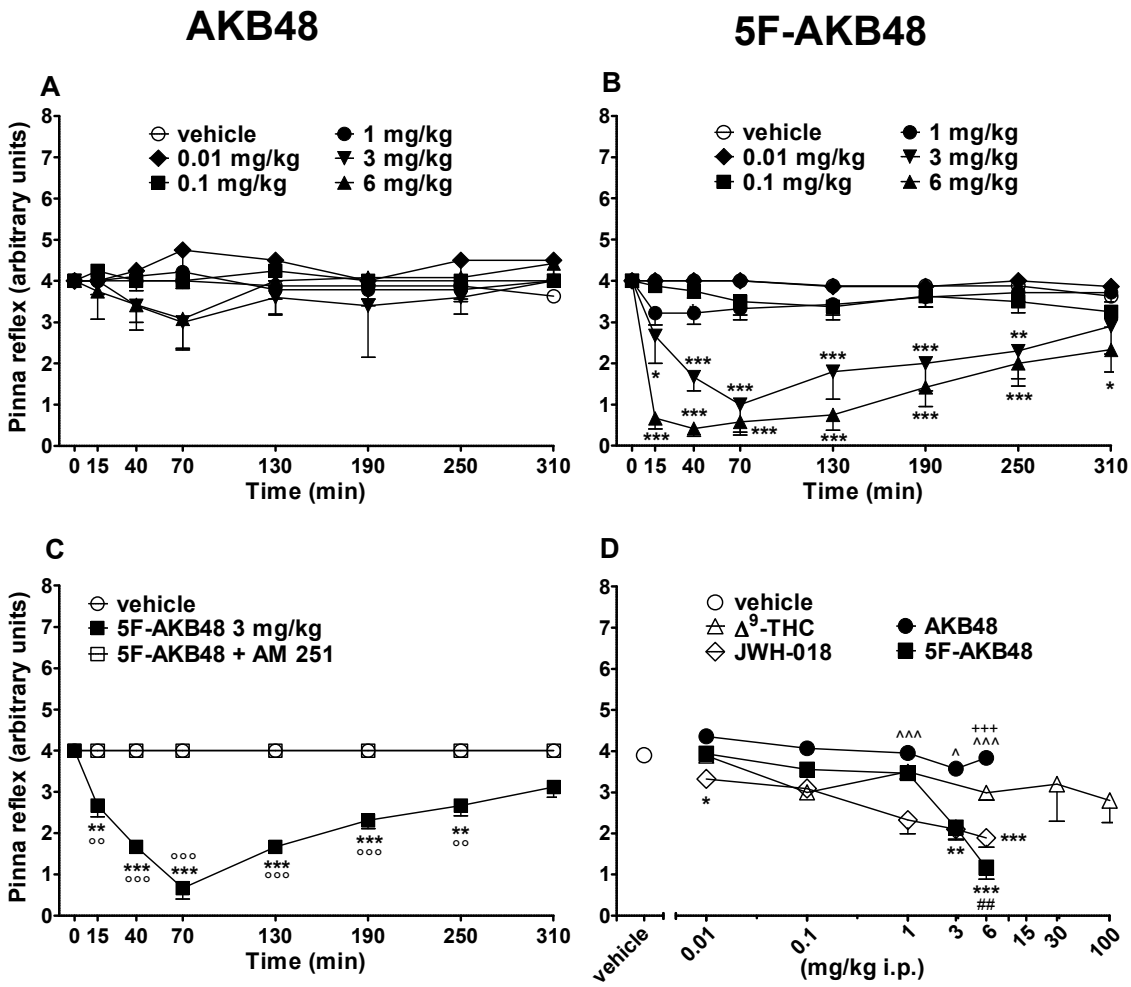
AKB48
5F-AKB48
A
B
C
D
vehicle
0.01 mg/kg
0.1 mg/kg
1 mg/kg
3 mg/kg
6 mg/kg
Pinna reflex (arbitrary units)
Time (min)
0 15 40 70 130 190 250 310
5F-AKB48 3 mg/kg
5F-AKB48 + AM 251
Δ9-THC
JWH-018
AKB48
5F-AKB48
(mg/kg i.p.)
vehicle 0.01 0.1 1 3 6 15 30 100

Figure 4

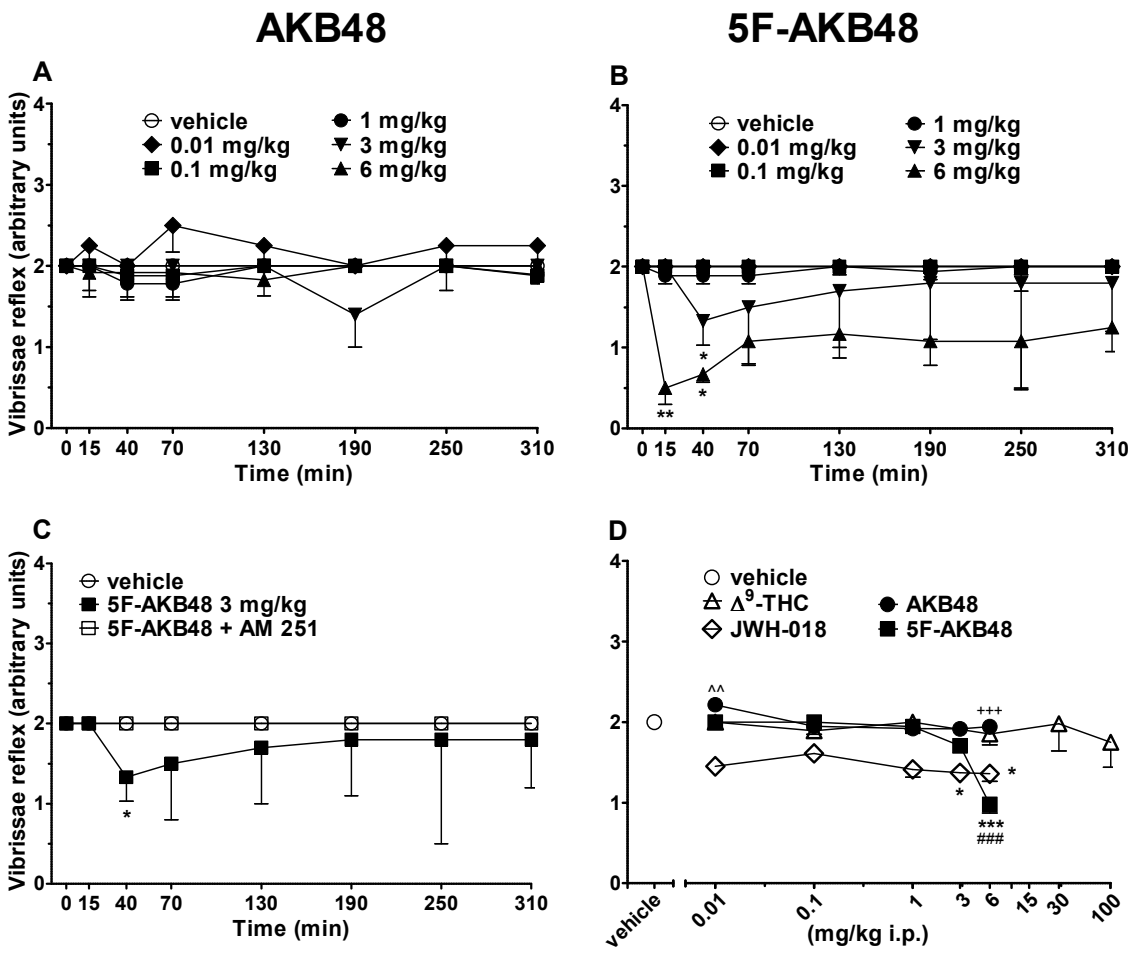
AKB48
5F-AKB48
A
B
C
D
vehicle
0.01 mg/kg
0.1 mg/kg
1 mg/kg
3 mg/kg
6 mg/kg
Vibrissae reflex (arbitrary units)
Time (min)
0 15 40 70 130 190 250 310
5F-AKB48 3 mg/kg
5F-AKB48 + AM 251
Δ9-THC
JWH-018
AKB48
5F-AKB48
(mg/kg i.p.)
vehicle 0.01 0.1 1 3 6 15 30 100

Figure 5

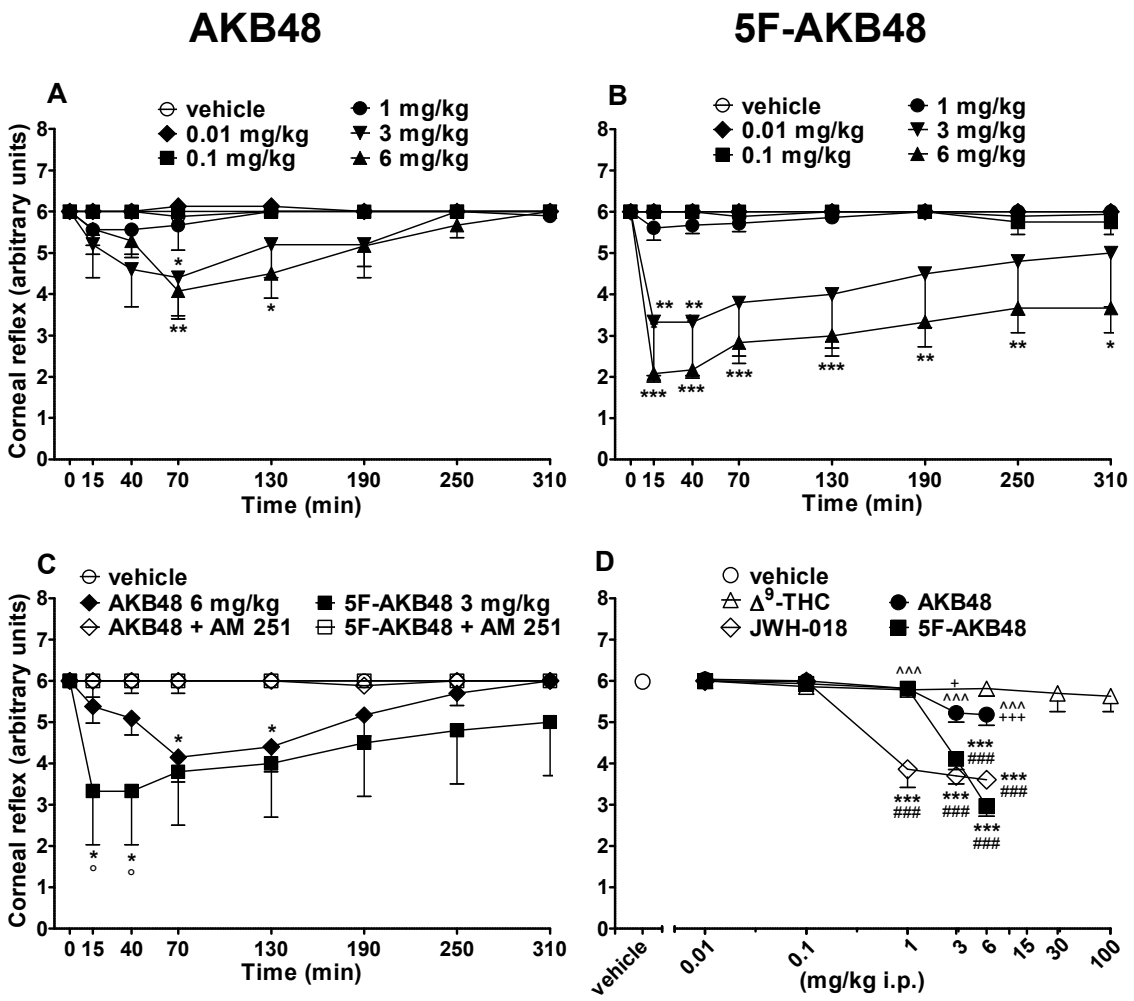
AKB48
5F-AKB48
A
B
C
D
vehicle
0.01 mg/kg
0.1 mg/kg
1 mg/kg
3 mg/kg
6 mg/kg
AKB48 6 mg/kg
AKB48 + AM 251
5F-AKB48 3 mg/kg
5F-AKB48 + AM 251
Δ9-THC
JWH-018
AKB48
5F-AKB48
Corneal reflex (arbitrary units)
Time (min)
(mg/kg i.p.)

Figure 6

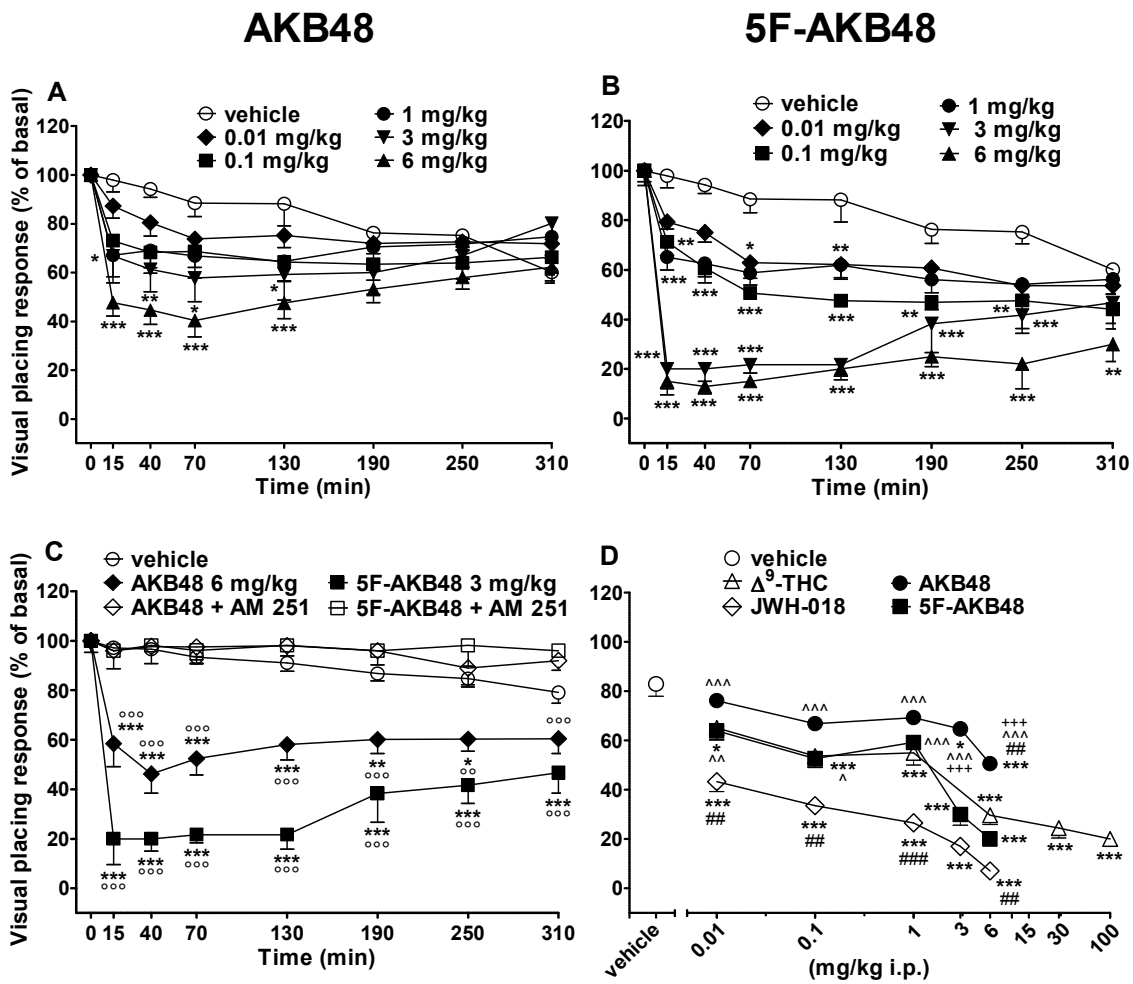
AKB48
5F-AKB48
A
B
C
D
vehicle
1 mg/kg
0.01 mg/kg
3 mg/kg
0.1 mg/kg
6 mg/kg
AKB48 6 mg/kg
5F-AKB48 3 mg/kg
AKB48 + AM 251
5F-AKB48 + AM 251
Δ9-THC
JWH-018
AKB48
5F-AKB48
Visual placing response (% of basal)
Time (min)
(mg/kg i.p.)

Figure 7

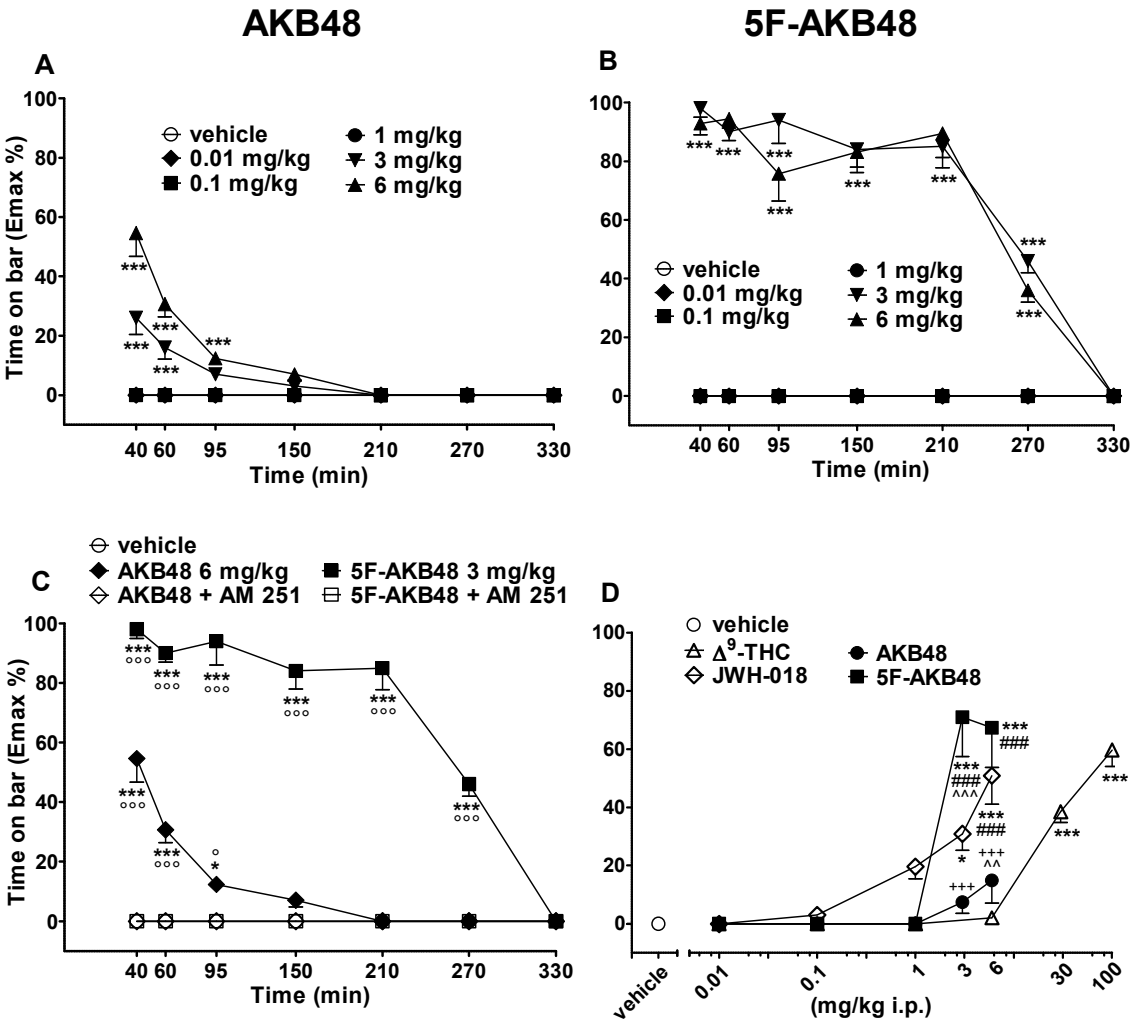
AKB48
5F-AKB48
A
B
C
D
vehicle
0.01 mg/kg
0.1 mg/kg
1 mg/kg
3 mg/kg
6 mg/kg
Time on bar (Emax %)
Time (min)
AKB48 6 mg/kg
AKB48 + AM 251
5F-AKB48 3 mg/kg
5F-AKB48 + AM 251
Δ9-THC
JWH-018
AKB48
5F-AKB48
(mg/kg i.p.)

Figure 8

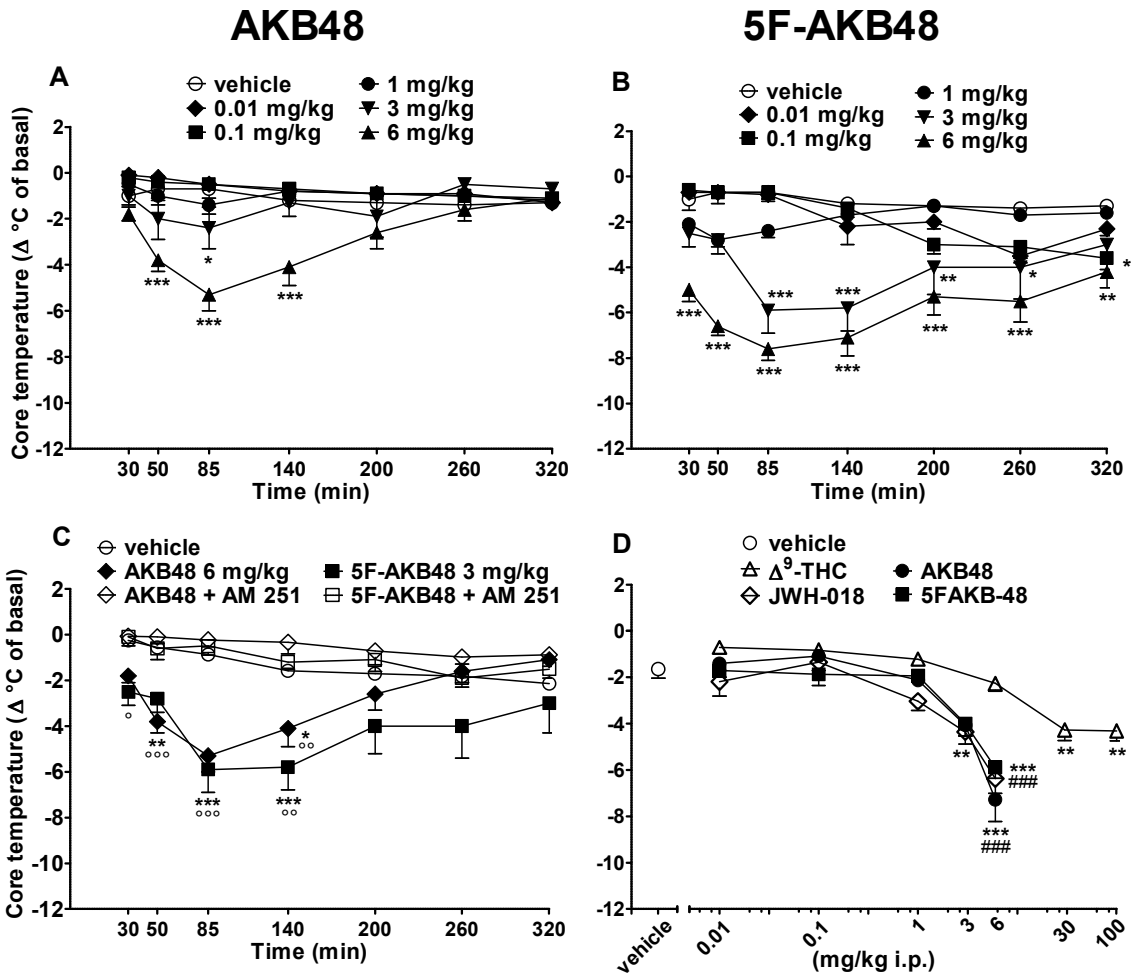
AKB48
5F-AKB48
A
B
C
D
vehicle
0.01 mg/kg
0.1 mg/kg
1 mg/kg
3 mg/kg
6 mg/kg
AKB48 6 mg/kg
AKB48 + AM 251
5F-AKB48 3 mg/kg
5F-AKB48 + AM 251
Δ9-THC
JWH-018
AKB48
5FAKB-48
Core temperature (Δ °C of basal)
Time (min)
(mg/kg i.p.)

Figure 9

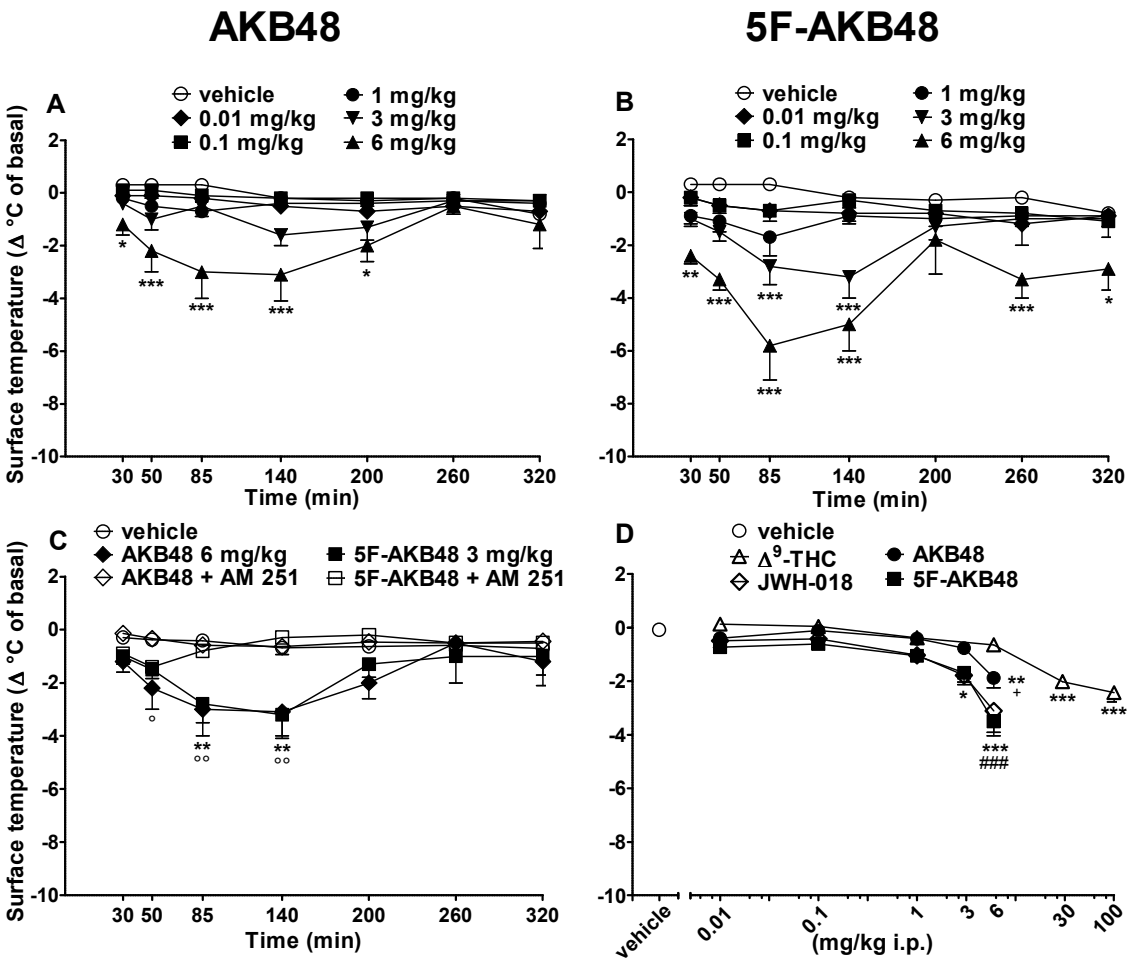
AKB48
5F-AKB48
A
B
C
D
vehicle
0.01 mg/kg
0.1 mg/kg
1 mg/kg
3 mg/kg
6 mg/kg
AKB48 6 mg/kg
AKB48 + AM 251
5F-AKB48 3 mg/kg
5F-AKB48 + AM 251
Δ9-THC
JWH-018
AKB48
5F-AKB48
Surface temperature (Δ °C of basal)
Time (min)
(mg/kg i.p.)

Figure 10

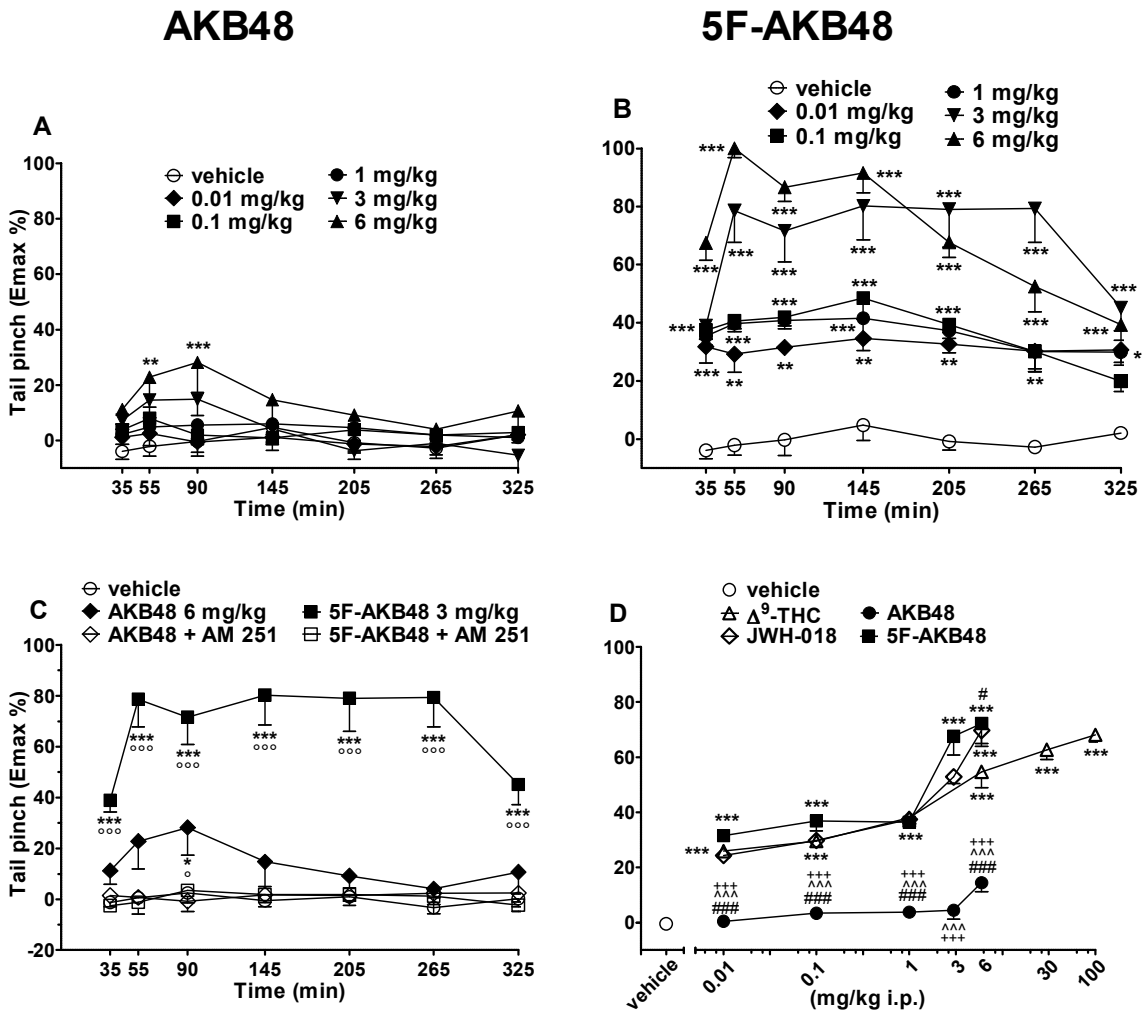
AKB48
5F-AKB48
A
B
C
D
vehicle
0.01 mg/kg
0.1 mg/kg
1 mg/kg
3 mg/kg
6 mg/kg
Tail pinch (Emax %)
Time (min)
AKB48 6 mg/kg
5F-AKB48 3 mg/kg
AKB48 + AM 251
5F-AKB48 + AM 251
Δ9-THC
JWH-018
AKB48
5F-AKB48
(mg/kg i.p.)

Figure 11

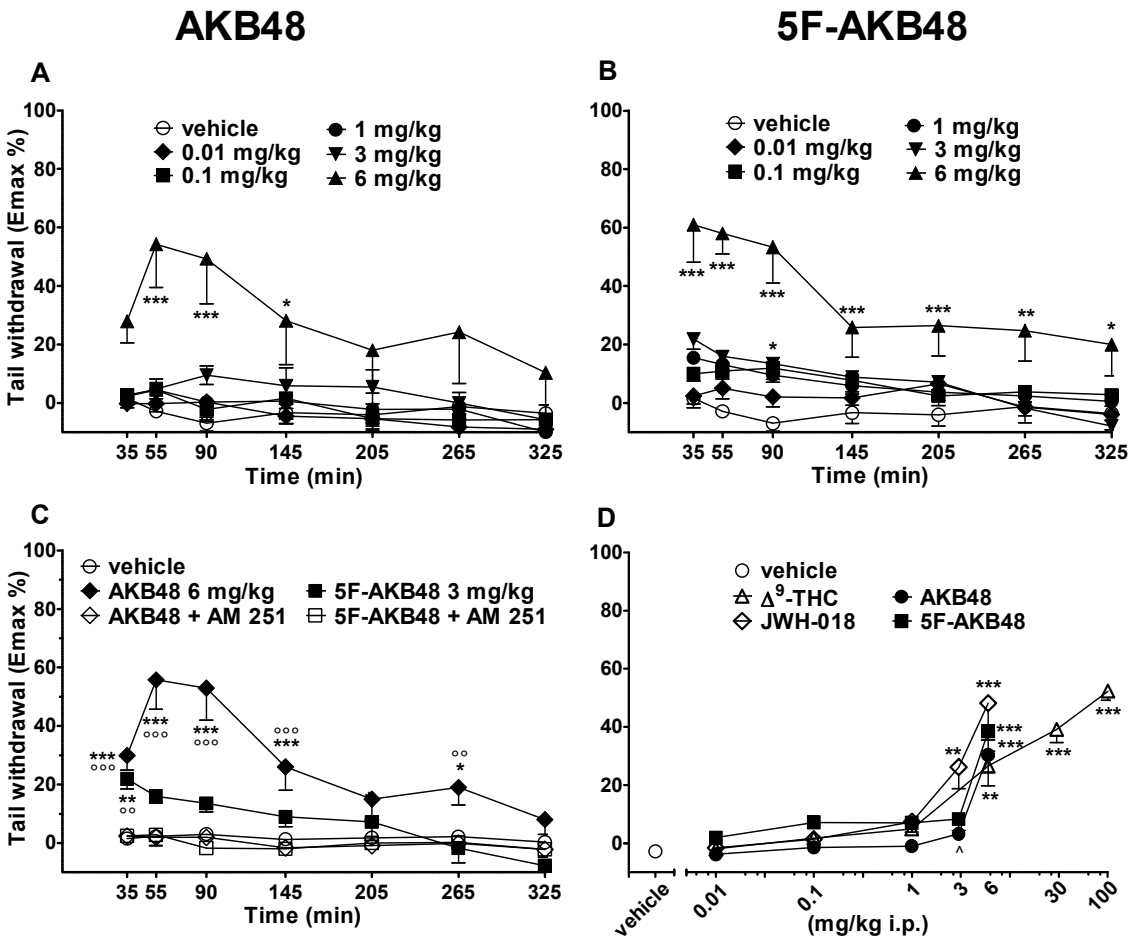
AKB48
5F-AKB48
A
B
C
D
vehicle
0.01 mg/kg
0.1 mg/kg
1 mg/kg
3 mg/kg
6 mg/kg
Tail withdrawal (Emax %)
Time (min)
35 55 90 145 205 265 325
AKB48 6 mg/kg
5F-AKB48 3 mg/kg
AKB48 + AM 251
5F-AKB48 + AM 251
Δ9-THC
JWH-018
AKB48
5F-AKB48
(mg/kg i.p.)
0.01 0.1 1 3 6 30 100

Figure 12

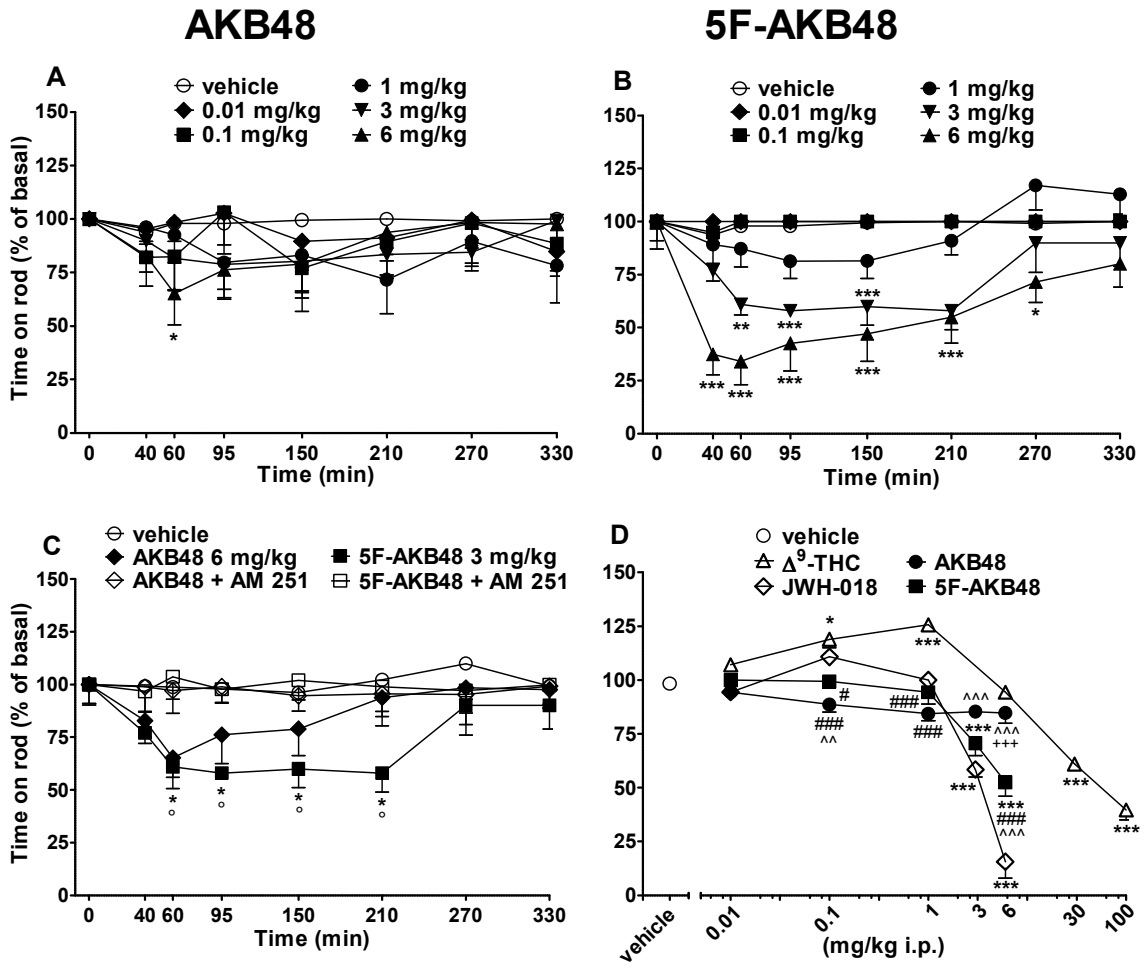
AKB48
5F-AKB48
A
B
C
D
vehicle
0.01 mg/kg
0.1 mg/kg
1 mg/kg
3 mg/kg
6 mg/kg
AKB48 6 mg/kg
AKB48 + AM 251
5F-AKB48 3 mg/kg
5F-AKB48 + AM 251
Δ9-THC
JWH-018
AKB48
5F-AKB48
Time on rod (% of basal)
Time (min)
(mg/kg i.p.)

Figure 13

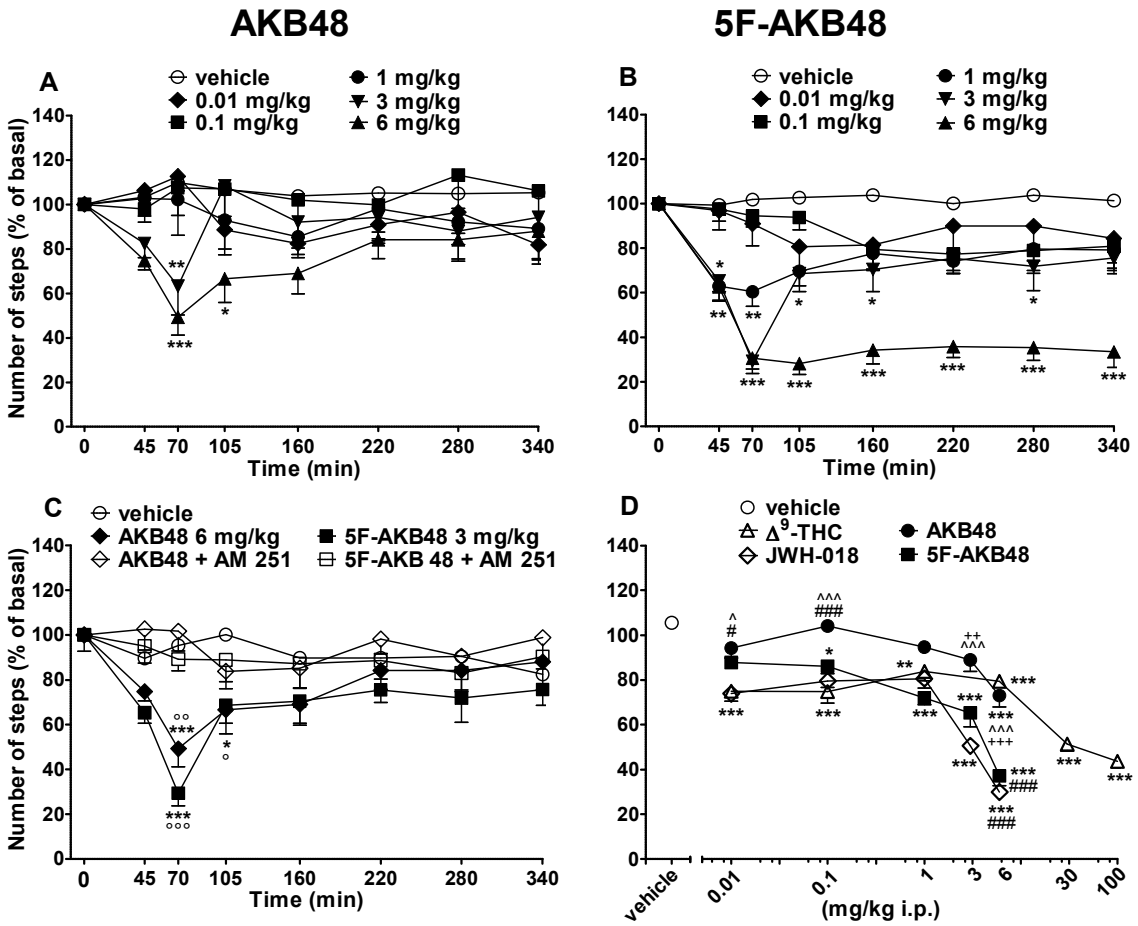
AKB48
5F-AKB48
A
B
C
D
vehicle
0.01 mg/kg
0.1 mg/kg
1 mg/kg
3 mg/kg
6 mg/kg
AKB48 6 mg/kg
AKB48 + AM 251
5F-AKB48 3 mg/kg
5F-AKB 48 + AM 251
Δ9-THC
JWH-018
AKB48
5F-AKB48
Number of steps (% of basal)
Time (min)
(mg/kg i.p.)

Figure 14

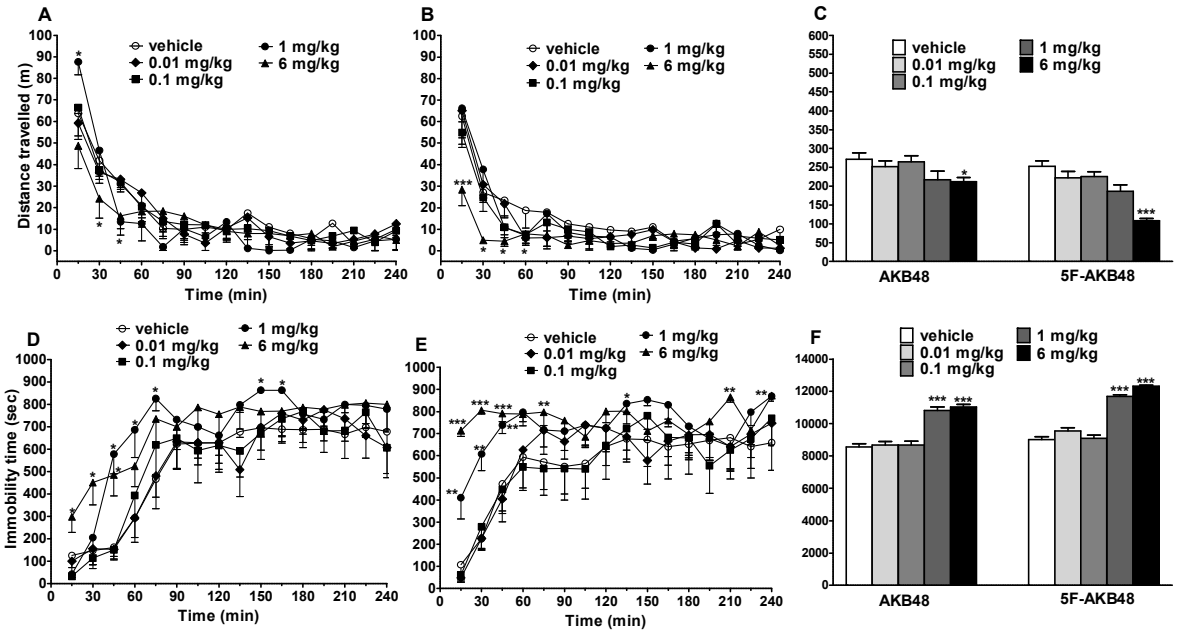
A
B
C
D
E
F
vehicle
0.01 mg/kg
0.1 mg/kg
1 mg/kg
6 mg/kg
Distance travelled (m)
Time (min)
Immobility time (sec)
AKB48
5F-AKB48

Figure 15

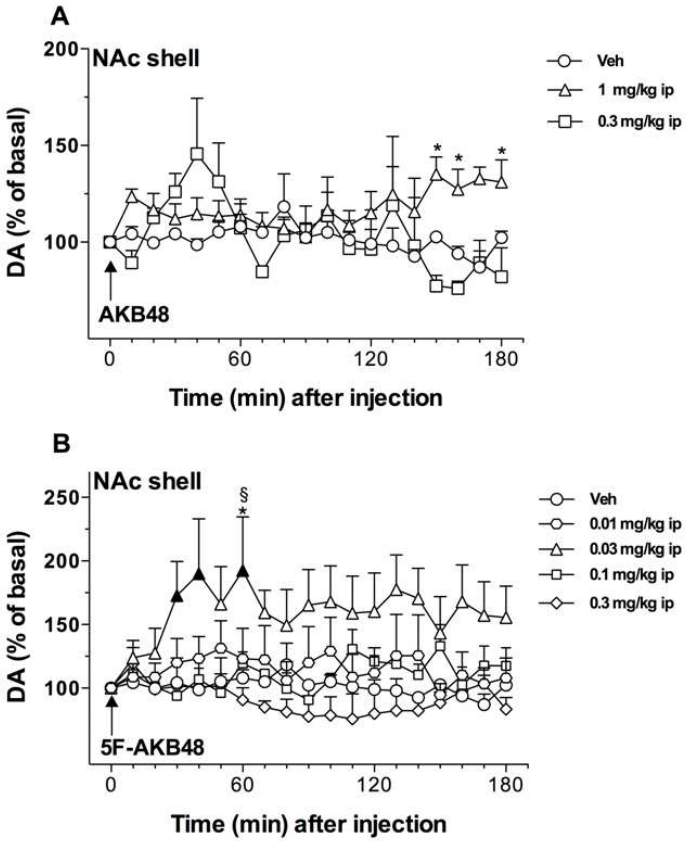
A
NAc shell
DA (% of basal)
200
150
100
AKB48
0
60
120
180
Time (min) after injection
Veh
1 mg/kg ip
0.3 mg/kg ip
*
*
*
B
NAc shell
DA (% of basal)
250
200
150
100
5F-AKB48
§
*
0
60
120
180
Time (min) after injection
Veh
0.01 mg/kg ip
0.03 mg/kg ip
0.1 mg/kg ip
0.3 mg/kg ip

Figure 16

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Figure captions

Figure 1 Competition curves of specific [^{3}H]-CP 55940 binding by AKB48 and 5F-AKB48 in CHO cell membranes transfected with human CB_1 receptors (A) or human CB_2 receptors (B) and to CB_1 receptors expressed in mouse brain membranes (C) or CB_2 receptors expressed in mouse spleen membranes (D). Inhibition curves of forskolin-stimulated cAMP accumulation by AKB48 and 5F-AKB48 in CHO cells transfected with human CB_1 receptors (E) or human CB_2 receptors (F). Results are given as the mean ± SEM of three independent experiments performed in duplicate

Figure 2 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the visual object test of mice. Interaction of effective dose of AKB48 and 5F-AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; #p<0.05, ###p<0.001 versus Δ^9-THC; ^p<0.05, ^^p<0.01, ^^^p<0.001 versus JWH-018, +p<0.05 versus 5F-AKB48 and °p<0.05, °°p<0.01, °°°p<0.001 versus AM 251+ agonist

Figure 3 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F-AKB48 (panel B) on the acoustic response test of mice. Interaction of effective dose of AKB48 and 5F-AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; #p<0.05,

##p<0.01, ###p<0.001 versus Δ^9-THC; ^^^p<0.001 versus JWH-018, +++p<0.001 versus 5F-AKB48 and °p<0.05, °°p<0.01, °°°p<0.001 versus AM 251+ agonist

Figure 4 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the pinna reflex of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; ##p<0.01 versus Δ^9-THC; ^p<0.05, ^^^p<0.001 versus JWH-018, +++p<0.001 versus 5F-AKB48 and °p<0.05, °°°p<0.001 versus AM 251+ agonist

Figure 5 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the vibrissae reflex of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compounds at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, ***p<0.001 versus vehicle; ###p<0.001 versus Δ^9-THC; ^^p<0.01 versus JWH-018 and +++p<0.001 versus 5F-AKB48

Figure 6 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the corneal reflex of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100

mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; ###p<0.001 versus Δ^9-THC; ^^^p<0.001 versus JWH-018, +p<0.05, +++p<0.001 versus 5F-AKB48 and °p<0.05 versus AM 251+ agonist

Figure 7 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the visual placing test of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; ##p<0.01, ###p<0.001 versus Δ^9-THC; ^p<0.05, ^^p<0.01, ^^^p<0.001 versus JWH-018, +++p<0.001 versus 5F-AKB48 and °°p<0.01, °°°p<0.001 versus AM 251+ agonist

Figure 8 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the bar test of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple

comparisons. *p<0.05, ***p<0.001 versus vehicle; ###p<0.001 versus Δ^9-THC; ^^p<0.01, ^^^p<0.001 versus JWH-018, +++p<0.001 versus 5F-AKB48 and °p<0.05, °°°p<0.001 versus AM 251+ agonist

Figure 9 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on mouse core temperature. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; ###p<0.001 versus Δ^9-THC; ^^^p<0.001 versus JWH-018, +++p<0.001 versus 5F-AKB48 and °p<0.05, °°p<0.01, °°°p<0.001 versus AM 251+ agonist

Figure 10 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the surface temperature of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; ###p<0.001 versus Δ^9-THC; +p<0.05 versus 5F-AKB48 and °p<0.05, °°p<0.01 versus AM 251+ agonist

Figure 11 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the tail pinch test of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C).

Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; #p<0.05, ###p<0.001 versus Δ^9-THC; ^^^p<0.001 versus JWH-018, +++p<0.001 versus 5F-AKB48 and °°°p<0.001 versus AM 251+ agonist

Figure 12 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the tail withdrawal test of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; ^p<0.05 versus JWH-018 and °°p<0.01, °°°p<0.001 versus AM 251+ agonist

Figure 13 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the accelerod test of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by

Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; #p<0.05, ###p<0.001 versus Δ^9-THC; ^^p<0.01, ^^^p<0.001 versus JWH-018, +++p<0.001 versus 5F-AKB48 and °p<0.05 versus AM 251+ agonist

Figure 14. Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the drag test of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; #p<0.05, ###p<0.001 versus Δ^9-THC; ^p<0.05, ^^^p<0.001 versus JWH-018, ++p<0.01, +++p<0.001 versus 5F-AKB48 and °p<0.05, °°p<0.01, °°°p<0.001 versus AM 251+ agonist

Figure 15 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A and D) and Δ^9-THC (panel B and E) on the total distance travelled and the total time immobile of the mouse. The overall effect observed in 5 hours (panel C and F) was also reported. Data are expressed as meters travelled (total distance travelled; panel A, B and C) and seconds of immobility (total time immobile; panel D, E and F) and represent the mean ± SEM of 10 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose response curve of AKB48 (panel A and D) and 5F-AKB48 (panel B and E) while the analysis of the overall effect (panel C and F) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. *p<0.05, ***p<0.001 versus vehicle

Figure 16 Effect of the systemic administration of AKB-48 (0.3, 1 mg/kg i.p.; panel A) and 5F-AKB48 (0.01-0.3 mg/kg i.p.; panel B) on DA transmission in the NAc shell of mice. Results are expressed as mean ± SEM of change in DA extracellular levels expressed as the percentage of basal values. Panel A: the arrow indicates the start of AKB-48 i.p. injection at the dose of 0.3 mg/kg (*squares*), 1 mg/kg (*triangles*), or vehicle (*circles*) in the NAc shell. Solid symbols: $p < 0.05$ with

respect to basal values; $^{*}p < 0.05$ vs 0.3 group; (NAc shell N=12) (Two-way ANOVA, Tukey's HSD post hoc). Panel B: the arrow indicates the start of 5F-AKB-48 i.p. injection at the dose of 0.01 mg/kg (*circles*), 0.03 mg/kg (*triangles*), 0.1 mg/kg (*squares*), 0.3 mg/kg (*diamonds*), or vehicle (*circles*) in the NAc shell. Solid symbol: $p < 0.05$ with respect to basal values; $^{§}p < 0.05$ vs 0.3 group; $^{*}p < 0.05$ vs veh; (NAc shell N=13) (Two-way ANOVA, Tukey's HSD post hoc)

Supplementary Material

Material and Methods

The protocol that we have used in this study is widely used in studies of "safety pharmacology" for the preclinical characterization of new molecules in rodents (Irwin 1968; Mattsson et al. 1996; Porsolt et al. 2002; Redfern et al. 2005; Hamdam et al. 2013; S7A 2001). Moreover, we previously validated this protocol to describe effects of cannabinoids on the “tetrad”, sensorimotor and neurological changes in mice (Vigolo et al. 2015; Ossato et al. 2015; Ossato et al. 2016). Additionally to substantiate the fact that our protocol causes a mild or no stress in animals we have compared and analyzed the behavioral motor, sensorimotor responses, nociceptive and body temperature changes in different groups of animals, in both naïve (untreated) and in treated animals with injection of saline or vehicle (Ossato et al. 2016 and present data). Despite the repetition of tests during the time, all animals have shown no changes in the parameters above described due to stress or discomfort. In particular, changes in body core temperature and responses to noxious stimuli, that are parameters sensitive to stressful situations (Adriaan Bouwknecht et al. 2007; Kozlov et al. 2015), have not shown any significant alteration in naïve animals and in those treated with saline or vehicle.

To reduce the number of animals used, the behaviour of mice were evaluated in five consecutive experimental sections carried out at different time period.

- First behavioral analysis performed in the time period 0-95 min
- Second behavioral analysis performed in the time period 120-150 min
- Third behavioral analysis performed in the time period 180-210 min
- Fourth behavioral analysis performed in the time period 240-270 min
- Fifth behavioral analysis performed in the time period 300-330 min

Each experimental section includes the following behavioral tests performed in a consecutive manner according to the following sequence: observation of main neurological changes and aggressive responses, measures of visual object responses (frontal and lateral view), acoustic response, tactile response (pinna, vibrissae and corneal reflexes) and visual placing response, evaluation of catalepsy, measures of core (rectal measurement), body temperature, determination of the mechanical (tail pinch) and thermal (tail withdrawal) acute pain and stimulated motor activity (accelerod and drag test).

In particular, to better understand the effects of SCBs in the first experimental section (0-95 min), tests were repeated consecutively, ensuring for each parameter three assessments, about once time every twenty minutes.

Moreover, between the first (0-95min) and the second (120-150 min) section, animals recovered 25 minutes while, between further sections, they rest 30 minutes (third sections 180-210 min; fourth session 240-270 min, fifth session 300-330 min).

During all sections of analysis, the period of rest between different tests was about 300 sec.

Behavioral tests

Neurological changes are expressed as frequency (percent of animals that develop symptoms), duration (total time in sec), latency (time in sec of symptom onset) and score (degree of tail elevation and number of bites connected to spontaneous and stimulated aggressiveness).

Visual Placing response test is performed using a tail suspension modified apparatus able to bring down the mouse towards the floor at a constant speed of 10 cm/sec (Ossato et al. 2015). The downward movement of the mouse is videotaped by a camera. The analysis frame by frame allows to evaluate the beginning of the reaction of the mouse while it is close to the floor. When the mouse beguines to react an electronic ruler evaluates the perpendicular distance in millimetres between the eyes of the mice and the floor. The mice untreated control perceives the floor and it prepares to contact at a distance of about 27 ± 4.5 mm. Evaluation of the visual placing response was measured at 0, 15, 35, 70, 125, 185, 245 and 305 min post injection.

Visual object response test was used to evaluate the ability of the mouse to see an object approaching from the front or the side, than inducing the animal to shift or turn the head or retreat it (Ossato et al. 2015). For the frontal visual response, a white horizontal bar was moved frontally to the mouse head and the manoeuvre was repeated 3 times. For the lateral visual response, a small dentist's mirror was moved into the mouse's field of view in an horizontal arc, until the stimulus was between the mouse's eyes. The procedure was conducted bilaterally and repeated 3 times. The score assigned was a value of 1 if there was a reflection in the mouse movement or 0 if not. The total value was calculated by adding the scores obtained in the frontal with that obtained in the lateral visual object response (overall score 9). Evaluation of the visual object response was measured at 0, 10, 30, 60, 120, 180, 240 and 300 min post injection.

Acoustic response measures the reflex of the mouse in replay to an acoustic stimulus produced behind the animal (Koch 1999). In particular, four acoustic stimuli of different intensity and frequency were tested (see Ossato et al. 2015). Each sound test was repeated 3 times, giving a value of 1 if there was a response, 0 if not present, for a total score of 3 for each sound. The acoustic total score was calculated by adding scores obtained in the four tests (overall score 12). Evaluation of the visual object response was measured at 0, 10, 30, 60, 120, 180, 240 and 300 min post injection.
The tactile response in the mouse was verified through vibrissae, pinna and corneal reflexes (Ossato et al. 2015). *Vibrissae reflex* was evaluated by touching vibrissae (right and left) with a thin hypodermic needle once for side giving a value of 1 if there was a reflex (turning of the head to the side of touch or vibrissae movement) or 0 if not present (overall score 2). Evaluation of the vibrissae reflex was measured at 0, 10, 30, 60, 120, 180, 240 and 300 min post injection. *Pinna reflex* was assessed by touching pavilions (left and right) with a thin hypodermic needle. First the interior pavilions and then the external were stimulated. This test was repeated twice for side giving a value of 1 if a reflex was present and 0 if not (overall score 4). Evaluation of the pinna reflex was measured at 0, 10, 30, 60, 120, 180, 240 and 300 min post injection. *Corneal reflex* was assessed gently touching the cornea of the mouse with a thin hypodermic needle and evaluating the response, assigning a value of 1 if the mouse moved only the head, 2 if it only closed the eyelid, 3 if it both closed the lid and moved the head. The procedure was conducted bilaterally (overall score 6) and was measured at 0, 10, 30, 60, 120, 180, 240 min post injection.

Core and surface mouse body temperatures were measured at 0, 30, 50, 85, 140, 200, 260 and 320 min post injection.

Acute mechanical nociception was evaluated using the tail pinch test (Vigolo et al. 2015). A special rigid probe connected to a digital dynamometer (ZP-50N, IMADA, Japan) was gently placed on the tail of the mouse (in the distal portion) and a progressive pressure was applied. When the mouse flicked its tail, the pressure was stopped and the digital instrument saved the maximum peak of weight supported (g/force). A cut off (500 g/force) was set to avoid tissue damage. The test was repeated three times and the final value was calculated with the average of 3 obtained scores. *Acute thermal nociception* was evaluated using the tail withdrawal test (Vigolo et al. 2015). The mouse was restrained in a dark plastic cylinder and half of its tail was dipped in water of 48°C and the time latency (in seconds) that the tail was left in water was recorded. A cut off (15 seconds) was set to avoid tissue damage. Acute mechanical and thermal nociception was measured at 0, 35, 55, 90, 145, 205, 265 and 325 min post injection.

Alterations of motor activity induced by AKB48 and 5F-AKB48 were measured using the bar, drag, accelerod tests and the analysis of spontaneous locomotor activity (Marti et al. 2004; Marti et al. 2005; Vigolo et al. 2015; Ossato et al. 2015). In the *bar test* each animal's forelimbs were placed on a bar made of plastic (height 6 cm). The time spent on the bar was measured (immobility cut off: 20 sec) and the akinesia was calculated as total time spent on the bar after three consecutive trials (total maximal time of catalepsy: 60 sec). The bar test was performed at 0, 20, 40, 75, 130, 190, 250 and 310 min post injection. In the *drag test,* the mouse was lifted by the tail, leaving the front paws on the table and dragged backward at a constant speed of about 20 cm/sec for a fixed distance (100 cm). The number of steps performed by each paw was recorded by two different observers. For each animal from five to seven measurements were collected. The drag test was performed at 0, 45, 70, 105, 160, 220, 280 and 340 min post injection. In the *accelerod test,* animals were placed on a rotating cylinder that increasing velocity automatically in a constant manner (0-60 rotations/min in 5 min). The time spent on the cylinder was measured. The accelerod test was performed at 0, 40, 60, 95, 150, 210, 270 and 330 min post injection. *Spontaneous locomotor activity* was measured by using the ANY-maze video-tracking system (Ugo Basile, application version 4.99g Beta). The mouse was placed in a square plastic cage (60 X 60 cm), located in a sound- and light-attenuated room, and motor activity was monitored for 240 min. Four mice were monitored at the same time in each experiment. Parameters measured were: distance travelled (m) and immobility time (sec; the animal is considered immobile when 95% of his image remains in the same place for at least 2 seconds). The distance covered and the time of immobility were analyzed every 15 minutes for a maximum of 240 minutes. To avoid mice olfactory cues, cages were carefully cleaned with a dilute (5%) ethanol solution and washed with water between animal trials. All experiments were performed between 9:00 AM to 1:00 PM.

References

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Statistical analysis

Table 2

Effects of the systemic administration of Δ^9-THC (0.01-100 mg/Kg i.p.), JWH-018, AKB48 and 5F-AKB48 (0.01-6 mg/Kg i.p.) on the neurological changes of the mouse.

Panel related to Convulsions: duration 6mgKg ($F_{2,29}$=9.290, p=0.0009) and latency 6mgKg ($F_{2,29}$=2.083, p=0.1441); *Panel related to Hyperreflexia*: duration 3 mgKg (t=1.497, df=18, P=0.1517) and latency 3 mgKg (t=1.104, df=18, P=0.2841); duration 6 mgKg ($F_{2,29}$=3.192, p=0.0584) and latency 6 mgKg ($F_{2,29}$=0.7683, p=0.4737); *Panel related to Myoclonias*: duration 1mgKg (t=0.8716, df=18, P=0.3949) and latency 1mgKg (t=0.1386, df=18, P=0.8913); duration 3mgKg (t=1.528, df=18, P=0.1438) and latency 3mgKg (t=0.6518, df=18, P=0.5227); duration 6mgKg ($F_{2,29}$=4.309, p=0.0238) and latency 6mgKg ($F_{2,29}$=4.191, p=0.0260); *Panel related to Elevation tail*: duration 3mgKg ($F_{2,29}$=1.264, p=0.2986), latency 3mgKg ($F_{2,29}$=0.08571, p=0.9181) and score 3mgKg ($F_{2,29}$=3.868, p=0.0333); duration 6mgKg ($F_{2,29}$=6.496, p=0.0050), latency 6mgKg ($F_{2,29}$=0.1842, p=0.8328) and score 6mgKg ($F_{2,29}$=4.707, p=0.0176); *Panel related to Spontaneous aggressiveness behaviour*: duration 3mgKg (t=0.3973, df=18, P=0.6958), latency 3mgKg (t=0.03400, df=18, P=0.9733) and score 3mgKg (t=0.02597, df=18, P=0.9796); duration 6mgKg ($F_{2,29}$=0.1902, p=0.8279), latency 6mgKg ($F_{2,29}$=0.02251, p=0.9778) and score 6mgKg ($F_{2,29}$=2.052, p=0.1480); *Panel related to Stimulated aggressiveness behaviour*: duration 3mgKg (t=15.66, df=18, P<0.0001) and score 3mgKg (t=1.394, df=18, P=0.1803); duration 6mgKg ($F_{2,29}$=73.35, p<0.0001) and score 6mgKg ($F_{2,29}$=1.255, p=0.3011);

Figure 2. Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the visual object test of mice. Interaction of effective dose of AKB48 and 5F-AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Panel A: significant effect of treatment ($F_{5,336}$=121.2, p<0.0001), time ($F_{7,336}$=58.03, p<0.0001) and time x treatment interaction ($F_{35,336}$=10.22, p<0.0001). Panel B: significant effect of treatment ($F_{5,336}$=165, p<0.0001), time ($F_{7,336}$=42.28, p<0.0001) and time x treatment interaction ($F_{35,336}$=5.751, p<0.0001). Panel C: significant effect of treatment ($F_{4,280}$=105.8, p<0.0001), time ($F_{7,280}$=12.71, p<0.0001) and time x treatment interaction ($F_{28,280}$=4.816, p<0.0001). Panel D: significant effect of AKB48 and 5F AKB48 versus Δ^9-THC and JWH-018 ($F_{21,175}$=53.23, p<0.0001).

Figure 3. Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F-AKB48 (panel B) on the acoustic response test of mice. Interaction of effective dose of AKB48 and 5F-AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Panel A: significant effect of treatment ($F_{5,336}$=20.33, p<0.0001), time ($F_{7,336}$=5.200, p<0.0001) and time x treatment interaction ($F_{35,336}$=1.616, p=0.0178). Panel B: significant effect of treatment ($F_{5,336}$=69.86, p<0.0001), time ($F_{7,336}$=8.506, p<0.0001) and time x treatment interaction ($F_{35,336}$=1.994, p=0.0001). Panel C: significant effect of treatment ($F_{4,280}$=44.50, p<0.0001), time ($F_{7,280}$=2.402, p=0.0211) and time x treatment interaction ($F_{28,280}$=0.9886, p=0.4857). Panel D: significant effect of AKB48 and 5F AKB48 versus Δ^9-THC and JWH-018 ($F_{21,175}$=26.08, p<0.0001).

Figure 4. Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the pinna reflex of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Panel A: significant effect of treatment ($F_{5,336}$=2.704, p=0.0206), time ($F_{7,336}$=0.3120, p=0.9484) and time x treatment interaction ($F_{35,336}$=0.5252, p=0.9887). Panel B: significant effect of treatment ($F_{5,336}$=73.21, p<0.0001), time ($F_{7,336}$=8.815, p<0.0001) and time x treatment interaction ($F_{35,336}$=2.829, p<0.0001). Panel C: significant effect of treatment ($F_{4,280}$=45.97, p<0.0001), time ($F_{7,280}$=4.720, p<0.0001) and time x treatment interaction ($F_{28,280}$=2.331, p=0.0003). Panel D: significant effect of AKB48 and 5F AKB48 versus Δ^9-THC and JWH-018 ($F_{21,175}$=8.692, p<0.0001).

Figure 5. Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the vibrissae reflex of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Panel A: significant effect of treatment ($F_{5,336}$=3.381, p=0.0054), time ($F_{7,336}$=0.6266, p=0.7339) and time x treatment interaction ($F_{35,336}$=0.6752, p=0.9210). Panel B: significant effect of treatment ($F_{5,336}$=10.70, p<0.0001), time ($F_{7,336}$=0.6608, p=0.7053) and time x treatment interaction ($F_{35,336}$=0.4060, p=0.9991). Panel C: significant effect of treatment ($F_{4,280}$=1.440, p=0.2208), time ($F_{7,280}$=0.08013, p=0.9992) and time x treatment

interaction ($F_{28,280}$=0.1442, p=1). Panel D: significant effect of AKB48 and 5F AKB48 versus Δ^9-THC and JWH-018 ($F_{21,175}$=6.524, p<0.0001).

Figure 6. Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the corneal reflex of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Panel A: significant effect of treatment ($F_{5,336}$=7.399, p<0.0001), time ($F_{7,336}$=2.313, p=0.0257) and time x treatment interaction ($F_{35,336}$=0.9600, p=0.5372). Panel B: significant effect of treatment ($F_{5,336}$=35.25, p<0.0001), time ($F_{7,336}$=2.886, p=0.0061) and time x treatment interaction ($F_{35,336}$=1.080, p=0.3538). Panel C: significant effect of treatment ($F_{4,280}$=12.61, p<0.0001), time ($F_{7,280}$=1.253, p=0.2738) and time x treatment interaction ($F_{28,280}$=0.6554, p=0.9103). Panel D: significant effect of AKB48 and 5F AKB48 versus Δ^9-THC and JWH-018 ($F_{21,175}$=23.30, p<0.0001).

Figure 7. Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the visual placing test of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Panel A: significant effect of treatment ($F_{5,336}$=16.59, p<0.0001), time ($F_{7,336}$=17.53, p<0.0001) and time x treatment interaction ($F_{35,336}$=1.509, p=0.0362). Panel B: significant effect of treatment ($F_{5,336}$=101.7, p<0.0001), time ($F_{7,336}$=57.26, p<0.0001) and time x treatment interaction ($F_{35,336}$=4.675, p<0.0001). Panel C: significant effect of treatment ($F_{4,280}$=175.4, p<0.0001), time ($F_{7,280}$=14.57, p<0.0001) and time x treatment interaction ($F_{28,280}$=5.088, p<0.0001). Panel D: significant effect of AKB48 and 5F AKB48 versus Δ^9-THC and JWH-018 ($F_{21,175}$=42.18, p<0.0001).

Figure 8. Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the bar test of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Panel A: significant effect of treatment ($F_{5,294}$=82.92, p<0.0001), time ($F_{6,294}$=47.54, p<0.0001) and time x treatment interaction ($F_{30,294}$=22.47, p<0.0001). Panel B: significant effect of treatment ($F_{5,294}$=875.6, p<0.0001), time ($F_{6,294}$=82.92, p<0.0001) and time x

treatment interaction ($F_{30,294}$=33.63, p<0.0001). Panel C: significant effect of treatment ($F_{4,245}$=838.5, p<0.0001), time ($F_{6,245}$=63.59, p<0.0001) and time x treatment interaction ($F_{24,245}$=37.93, p<0.0001). Panel D: significant effect of AKB48 and 5F AKB48 versus Δ^9-THC and JWH-018 ($F_{21,175}$=21.19, p<0.0001).

Figure 9. Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on mouse core temperature. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Panel A: significant effect of treatment ($F_{5,294}$=30.31, p<0.0001), time ($F_{6,294}$=4.155, p=0.0005) and time x treatment interaction ($F_{30,294}$=3.316, p<0.0001). Panel B: significant effect of treatment ($F_{5,294}$=69.51, p<0.0001), time ($F_{6,294}$=3.690, p=0.0015) and time x treatment interaction ($F_{30,294}$=3.208, p<0.0001). Panel C: significant effect of treatment ($F_{4,245}$=41.91, p<0.0001), time ($F_{6,245}$=4.654, p=0.0002) and time x treatment interaction ($F_{24,245}$=2.527, p=0.0002). Panel D: significant effect of AKB48 and 5F AKB48 versus Δ^9-THC and JWH-018 ($F_{21,175}$=19.45, p<0.0001).

Figure 10. Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the surface temperature of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Panel A: significant effect of treatment ($F_{5,294}$=22.80, p<0.0001), time ($F_{6,294}$=2.966, p=0.0079) and time x treatment interaction ($F_{30,294}$=1.598, p=0.0280). Panel B: significant effect of treatment ($F_{5,294}$=38.61, p<0.0001), time ($F_{6,294}$=3.816, p=0.0011) and time x treatment interaction ($F_{30,294}$=1.740, p=0.0116). Panel C: significant effect of treatment ($F_{4,245}$=15.09, p<0.0001), time ($F_{6,245}$=3.481, p=0.0025) and time x treatment interaction ($F_{24,245}$=1.435, p=0.0913). Panel D: significant effect of AKB48 and 5F AKB48 versus Δ^9-THC and JWH-018 ($F_{21,175}$=12.99, p<0.0001).

Figure 11. Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the tail pinch test of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Panel A: significant effect of treatment ($F_{5,294}$=8.163,

p<0.0001), time ($F_{6,294}$=2.481, p=0.0234) and time x treatment interaction ($F_{30,294}$=0.8253, p=0.7306). Panel B: significant effect of treatment ($F_{5,294}$=132.6, p<0.0001), time ($F_{6,294}$=10.34, p<0.0001) and time x treatment interaction ($F_{30,294}$=2.854, p<0.0001). Panel C: significant effect of treatment ($F_{4,245}$=140.6, p<0.0001), time ($F_{6,245}$=2.460, p=0.0250) and time x treatment interaction ($F_{24,245}$=1.735, p=0.0206). Panel D: significant effect of AKB48 and 5F AKB48 versus Δ^9-THC and JWH-018 ($F_{21,175}$=52.10 p<0.0001).

Figure 12. Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the tail withdrawal test of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Panel A: significant effect of treatment ($F_{5,294}$=24.39, p<0.0001), time ($F_{6,294}$=3.051, p=0.0065) and time x treatment interaction ($F_{30,294}$=0.7765, p=0.7965). Panel B: significant effect of treatment ($F_{5,294}$=53.83, p<0.0001), time ($F_{6,294}$=9.265, p<0.0001) and time x treatment interaction ($F_{30,294}$=1.737, p=0.0118). Panel C: significant effect of treatment ($F_{4,245}$=64.34, p<0.0001), time ($F_{6,245}$=10.50, p<0.0001) and time x treatment interaction ($F_{24,245}$=3.992, p<0.0001). Panel D: significant effect of AKB48 and 5F AKB48 versus Δ^9-THC and JWH-018 ($F_{21,175}$=16.90, p<0.0001).

Figure 13. Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the accelerod test of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Panel A: significant effect of treatment ($F_{5,336}$=1.570, p<0.1678), time ($F_{7,336}$=1.084, p=0. 3729) and time x treatment interaction ($F_{35,336}$=0.4796, p=0.9950). Panel B: significant effect of treatment ($F_{5,336}$=49.59, p<0.0001), time ($F_{7,336}$=9.236, p<0.0001) and time x treatment interaction ($F_{35,336}$=2.442, p<0.0001). Panel C: significant effect of treatment ($F_{4,280}$=10.69, p<0.0001), time ($F_{7,280}$=1.928, p=0.0652) and time x treatment interaction ($F_{28,280}$=0.8168, p=0.7335). Panel D: significant effect of AKB48 and 5F AKB48 versus Δ^9-THC and JWH-018 ($F_{21,175}$=51.44, p<0.0001).

Figure 14. Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the drag test of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB_1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison

of the total average effect observed in 5 hours (panel D) with Δ^9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Panel A: significant effect of treatment ($F_{5,336}$=8.390, p<0.0001), time ($F_{7,336}$=0.6685, p=0.6988) and time x treatment interaction ($F_{35,336}$=1.102, p=0.3230). Panel B: significant effect of treatment ($F_{5,336}$=52.04, p<0.0001), time ($F_{7,336}$=9.090, p<0.0001) and time x treatment interaction ($F_{35,336}$=2.451, p<0.0001). Panel C: significant effect of treatment ($F_{4,280}$=15.91, p<0.0001), time ($F_{7,280}$=4.881, p<0.0001) and time x treatment interaction ($F_{28,280}$=1.827, p=0.0081). Panel D: significant effect of AKB48 and 5F AKB48 versus Δ^9-THC and JWH-018 ($F_{21,175}$=33.20, p<0.0001).

Figure 15. Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A and D) and 5F AKB48 (panel B and E) on the total distance travelled and the total time immobile of mice. The overall effect observed in 5 hours (panel C and F) was also reported. Panel A: significant effect of treatment ($F_{4,720}$=1.735, p=0.1157), time ($F_{15,720}$=45.22, p<0.0001) and time x treatment interaction ($F_{60,720}$=1.235, p=0.1404). Panel B: significant effect of treatment ($F_{4,720}$=5.381, p=0.003), time ($F_{15,720}$=30.83, p<0.0001) and time x treatment interaction ($F_{60,720}$=2.071, p=1.234). Panel C: significant effect of AKB48 and 5F AKB48 ($F_{9,99}$=9.530, p<0.0001); Panel D: significant effect of treatment ($F_{4,720}$=12.06, p<0.0001), time ($F_{15,720}$=25.20, p<0.0001) and time x treatment interaction ($F_{60,720}$=0.6346, p=0.9858). Panel E: significant effect of treatment ($F_{4,720}$=15.84, p<0.0001), time ($F_{15,720}$=9.278, p<0.0001) and time x treatment interaction ($F_{60,720}$=0.9946, p=0.4900). Panel F: significant effect of AKB48 and 5F AKB48 ($F_{9,99}$=54.47, p<0.0001).

Figure 16. Effect of AKB48 (0.3 and 1 mg/kg i.p.) and 5F-AKB48 (0.01, 0.03, 0.1 and 0.3 mg/kg/i.p.) on extracellular DA release in NAc shell of awake and freely moving mice (panel A and B). Panel A: significant effect of treatment ($F_{2,9}$=1.86, p>0.05), time ($F_{18,162}$=1.17, p>0.05) and time x treatment interaction ($F_{36,162}$=2.14, *p<0.001); Panel B: significant effect of treatment ($F_{3,9}$=4.59, *p<0.05), time ($F_{18,162}$=0.91, p>0.05) and time x treatment interaction ($F_{54,162}$=1.46, *p<0.05).

Table 1

Binding and functional parameters of AKB48 and 5F-AKB48 to human and mouse CB_1 and CB_2 receptors

Compound	*hCB1 CHO membranesa[a] Ki (nM)*	*hCB2 CHO membranes[a] Ki (nM)*	*Mouse cortex membranes CB1[a] Ki (nM)*	*Mouse spleen membranes CB2[a] Ki (nM)*	*hCB1 CHO cells[b] IC50 (nM)*	*hCB2 CHO cells[b] IC50 (nM)*
AKB48	3.24 ± 0.28	1.68 ± 0.12	5.34 ± 0.44	1.93 ± 0.14	5.39 ± 0.47	2.13 ± 0.21
5F-AKB48	1.82 ± 0.15	0.82 ± 0.07	3.87 ± 0.27	1.24 ± 0.07	2.57 ± 0.19	1.94 ± 0.14

Data are expressed as mean ± SEM.
[a] *[^{3}H]-CP-55,940 competition binding experiments.*
[b] *Cyclic AMP experiments.*

Table 2

Effects of the systemic administration of Δ^9-THC (0.01-100 mg/Kg i.p.), JWH-018, AKB48 and 5F-AKB48 (0.01-6 mg/Kg i.p.) on the neurological changes of the mouse.

Elevation tail

Compound	Vehicle	Δ^9-THC[a]							JWH-018[a]					AKB48					5F-AKB48				
Doses (mg/Kg)		0.01	0.1	1	3	6	30	100	0.01	0.1	1	3	6	0.01	0.1	1	3	6	0.01	0.1	1	3	6
Frequency (%)	–	–	–	–	–	–	10	65	–	–	10	35	80	–	–	–	40	70	–	–	–	30	75
Score	–	–	–	–	–	–	0.6±0.76	1.1±0.8	–	–	0.5±0.41	1.2±0.17	2.4±0.33	–	–	–	2.2±0.6	1.88±0.3	–	–	–	2.6±0.12 *	3.1±0.2 #
Duration (sec)	–	–	–	–	–	–	654.7±82.9	934.7±88.2	–	–	412.6±132.9	1236.8±111.5	1766.6±189.7	–	–	–	1007.7±146	998.5±89 **	–	–	–	1271±122	1436.1±157
Latency (sec)	–	–	–	–	–	–	112.5±33.9	103.6±17.4	–	–	92.5±17.1	94.8±16.3	88.6±13.4	–	–	–	99.7±24	101.2±21	–	–	–	88.2±18	91.2±10

Hyperrilexia

Compound	Vehicle	Δ^9-THC[a]							JWH-018[a]					AKB48					5F-AKB48				
Doses (mg/Kg)		0.01	0.1	1	3	6	30	100	0.01	0.1	1	3	6	0.01	0.1	1	3	6	0.01	0.1	1	3	6
Frequency (%)	–	–	–	–	–	–	–	–	–	–	–	15	80	–	–	–	–	25	–	–	–	30	75
Duration (sec)	–	–	–	–	–	–	–	–	–	–	–	1354.8±67.2	1439.8±45.3	–	–	–	–	1980.2±298	–	–	–	1009±221	1254.2±210
Latency (sec)	–	–	–	–	–	–	–	–	–	–	–	124.9±31.7	93.5±21.2	–	–	–	–	143±55	–	–	–	191.2±51	179±61

Myoclonie

Compound	Vehicle	Δ^9-THC[a]							JWH-018[a]					AKB48					5F-AKB48				
Doses (mg/Kg)		0.01	0.1	1	3	6	30	100	0.01	0.1	1	3	6	0.01	0.1	1	3	6	0.01	0.1	1	3	6
Frequency (%)	–	–	–	–	–	–	–	–	–	–	–	–	80	–	–	–	–	45	–	–	–	90	100
Duration (sec)	–	–	–	–	–	–	–	–	–	–	–	–	669.7±36.6	–	–	–	–	1087.6±241	–	–	–	1181.6±224	1593±299 *
Latency (sec)	–	–	–	–	–	–	–	–	–	–	–	–	109.7±16.35	–	–	–	–	185±19.7	–	–	–	224±41	210±36 *

Convulsion

Compound	Vehicle	Δ^9-THC[a]							JWH-018[a]					AKB48					5F-AKB48				
Doses (mg/Kg)		0.01	0.1	1	3	6	30	100	0.01	0.1	1	3	6	0.01	0.1	1	3	6	0.01	0.1	1	3	6
Frequency (%)	–	–	–	–	–	–	–	–	–	–	–	–	70	–	–	–	–	10	–	–	–	30	90
Duration (sec)	–	–	–	–	–	–	–	–	–	–	–	–	369.7±32.2	–	–	–	–	454.2±67.1	–	–	–	480.1±59	1821.3±457 ** ##
Latency (sec)	–	–	–	–	–	–	–	–	–	–	–	–	109.7±16.3	–	–	–	–	248±79	–	–	–	271.3±47.9	274.7±69.2

Spontaneous aggressiveness

Compound	Vehicle	Δ^9-THC[b]							JWH-018[b]					AKB48					5F-AKB48				
Doses (mg/Kg)		0.01	0.1	1	3	6	30	100	0.01	0.1	1	3	6	0.01	0.1	1	3	6	0.01	0.1	1	3	6
Frequency (%)	–	–	–	–	–	–	–	–	–	–	–	–	90	–	–	–	–	50	–	–	–	50	100
Score (n° of bites)	–	–	–	–	–	–	–	–	–	–	–	–	8.2±3.1	–	–	–	–	1.60±1.7	–	–	–	1.4±0.31	1.7±2.9
Duration (sec)	–	–	–	–	–	–	–	–	–	–	–	–	2750±621	–	–	–	–	2971.1±581	–	–	–	3303±512	3272.6±602
Latency (sec)	–	–	–	–	–	–	–	–	–	–	–	–	324.5±76	–	–	–	–	318.2±88	–	–	–	296.2±66	302.2±64

Stimulated aggressiveness

Compound	Vehicle	Δ^9-THC							JWH-018					AKB48					5F-AKB48				
Doses (mg/Kg)		0.01	0.1	1	3	6	30	100	0.01	0.1	1	3	6	0.01	0.1	1	3	6	0.01	0.1	1	3	6
Frequency (%)	–	–	–	–	–	–	–	–	–	–	–	50	100	–	–	–	35	70	–	–	30	70	100
Score (n° of bites)	–	–	–	–	–	–	–	–	–	–	–	5.1±4.3	10±0.2	–	–	–	1.75±1.7	4.37±3.9	–	–	3.75±4.1	7.3±3.6	4.75±2.9
Duration (sec)	–	–	–	–	–	–	–	–	–	–	–	12316±420	15286±408	–	–	–	3499±553	7217.1±677 ***	–	–	3051.8±590	14071±387 ###	14482±428 ###

Data are expressed as percentage (frequency of animal with neurological signs), seconds (duration and latency of neurological signs) and score (number of bites connected to spontaneous and stimulated aggressiveness and degree of elevation connected to the elevation tail), represent the mean ± SEM of 10 animals for each treatment. Statistical analysis was performed with one-way ANOVA followed by Tukey's test for multiple comparisons and Student's t-test was used to determine statistical significance ($P<0.05$) between two groups. *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus JWH-018 at the same dosage and #$p<0.05$, ##$p<0.01$, ###$p<0.001$ versus AKB48 at the same dosage.

[a] *from Vigolo et al. 2015*

[b] *from Ossato et al. 2016*

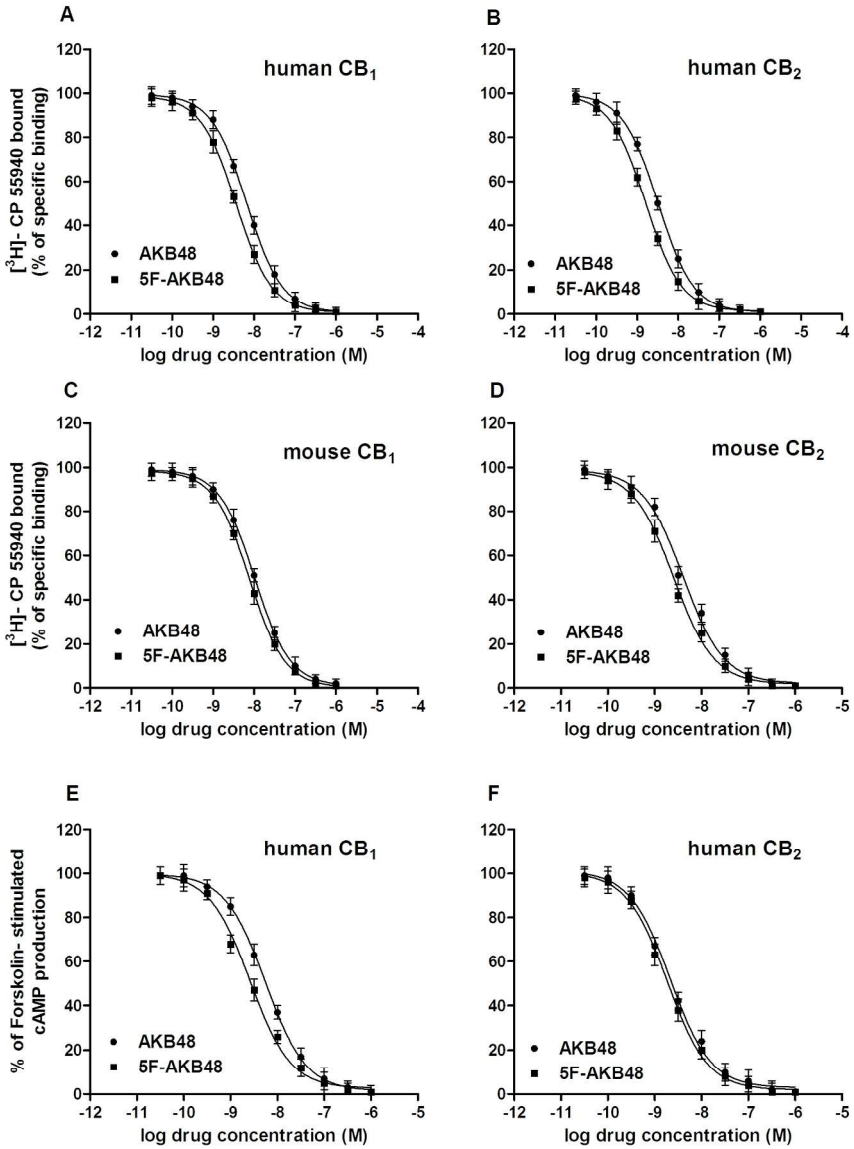

Figure 1 Competition curves of specific [3H]-CP 55940 binding by AKB48 and 5F-AKB48 in CHO cell membranes transfected with human CB1 receptors (A) or human CB2 receptors (B) and to CB1 receptors expressed in mouse brain membranes (C) or CB2 receptors expressed in mouse spleen membranes (D). Inhibition curves of forskolin-stimulated cAMP accumulation by AKB48 and 5F-AKB48 in CHO cells transfected with human CB1 receptors (E) or human CB2 receptors (F). Results are given as the mean ± SEM of three independent experiments performed in duplicate
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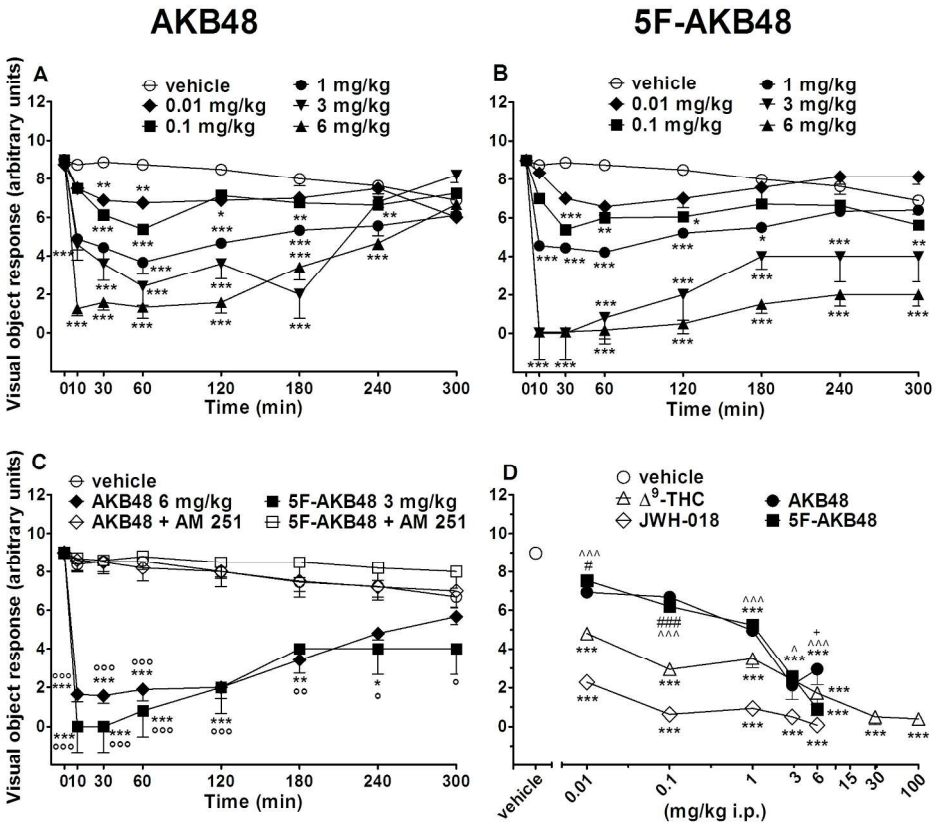

Figure 2 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the visual object test of mice. Interaction of effective dose of AKB48 and 5F-AKB48 (6 mg/kg) with the selective CB1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey\'s test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; #p<0.05, ###p<0.001 versus Δ9-THC; ^p<0.05, ^^p<0.01, ^^^p<0.001 versus JWH-018, +p<0.05 versus 5F-AKB48 and °p<0.05, °°p<0.01, °°°p<0.001 versus AM 251+ agonist
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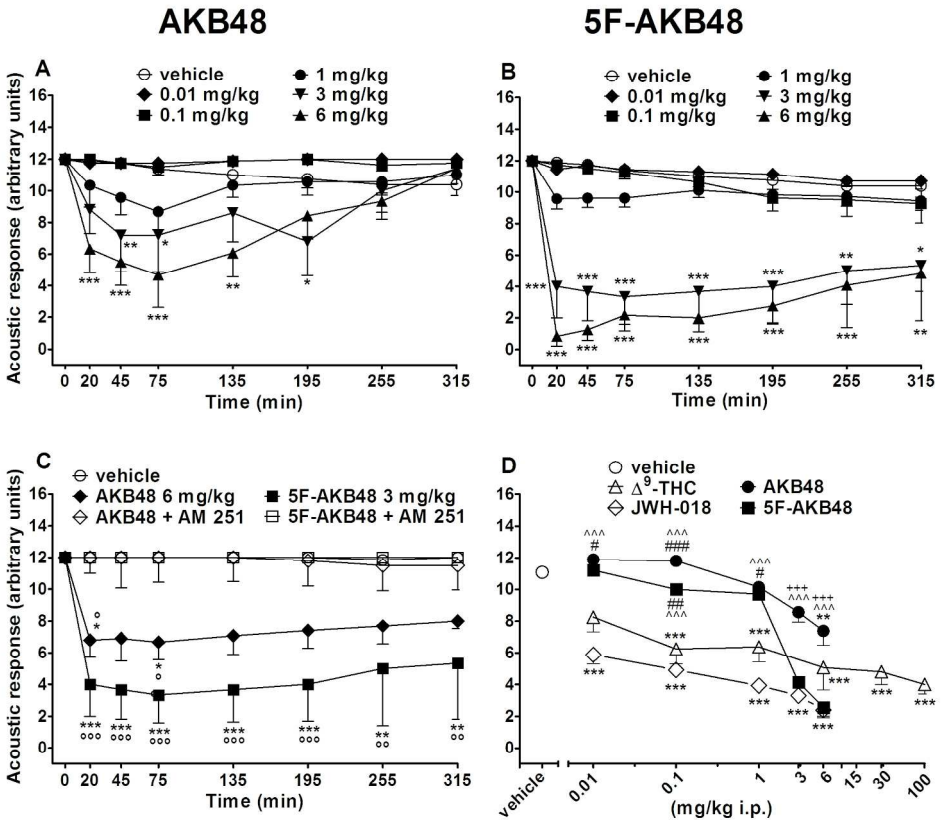

Figure 3 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F-AKB48 (panel B) on the acoustic response test of mice. Interaction of effective dose of AKB48 and 5F-AKB48 (6 mg/kg) with the selective CB1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey\'s test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; #p<0.05, ##p<0.01, ###p<0.001 versus Δ9-THC; ^^^p<0.001 versus JWH-018, +++p<0.001 versus 5F-AKB48 and °p<0.05, °°p<0.01, °°°p<0.001 versus AM 251+ agonist
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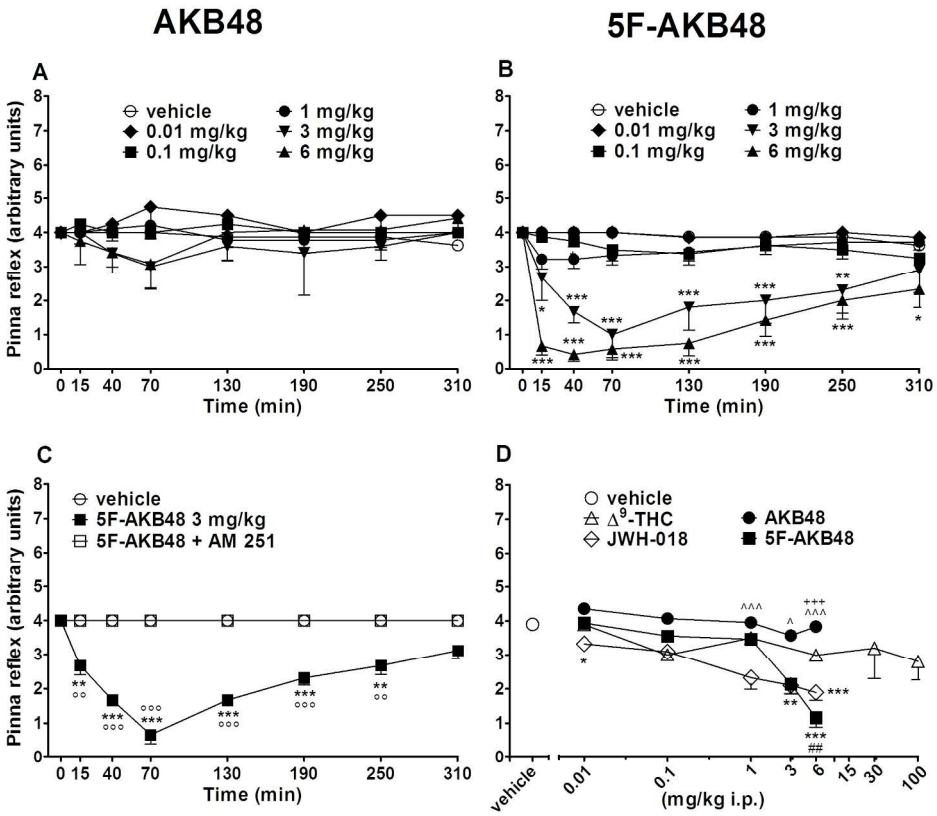

Figure 4 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the pinna reflex of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey\'s test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; ##p<0.01 versus Δ9-THC; ^p<0.05, ^^^p<0.001 versus JWH-018, +++p<0.001 versus 5F-AKB48 and °p<0.05, °°°p<0.001 versus AM 251+ agonist
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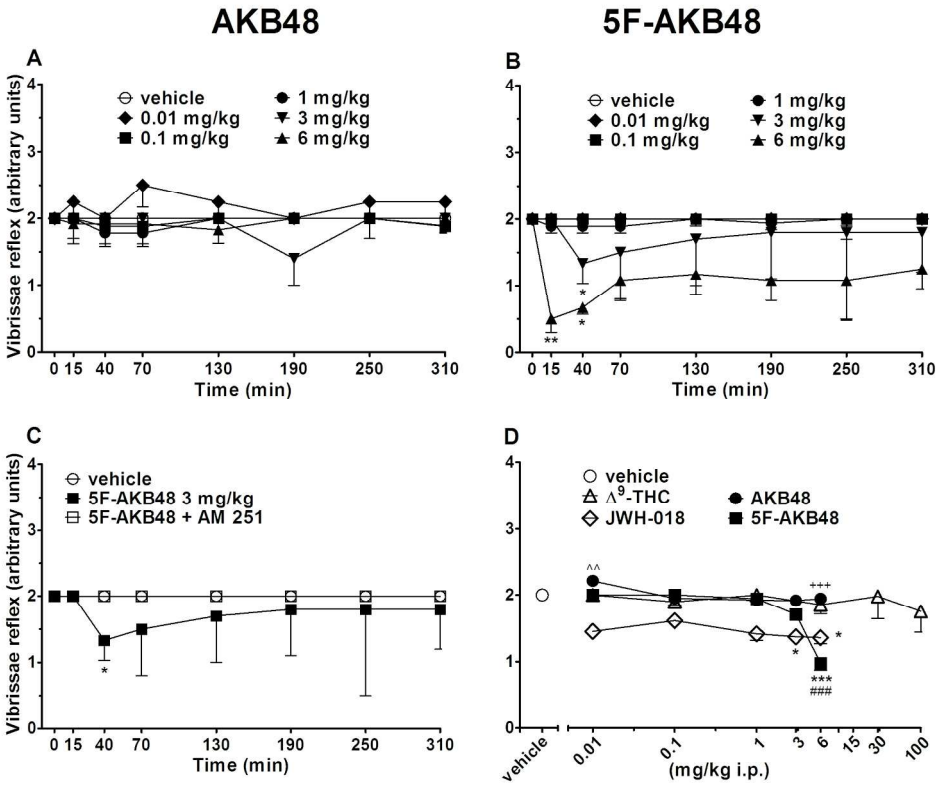

Figure 5 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the vibrissae reflex of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compounds at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey\'s test for multiple comparisons. *p<0.05, ***p<0.001 versus vehicle; ###p<0.001 versus Δ9-THC; ^^p<0.01 versus JWH-018 and +++p<0.001 versus 5F-AKB48
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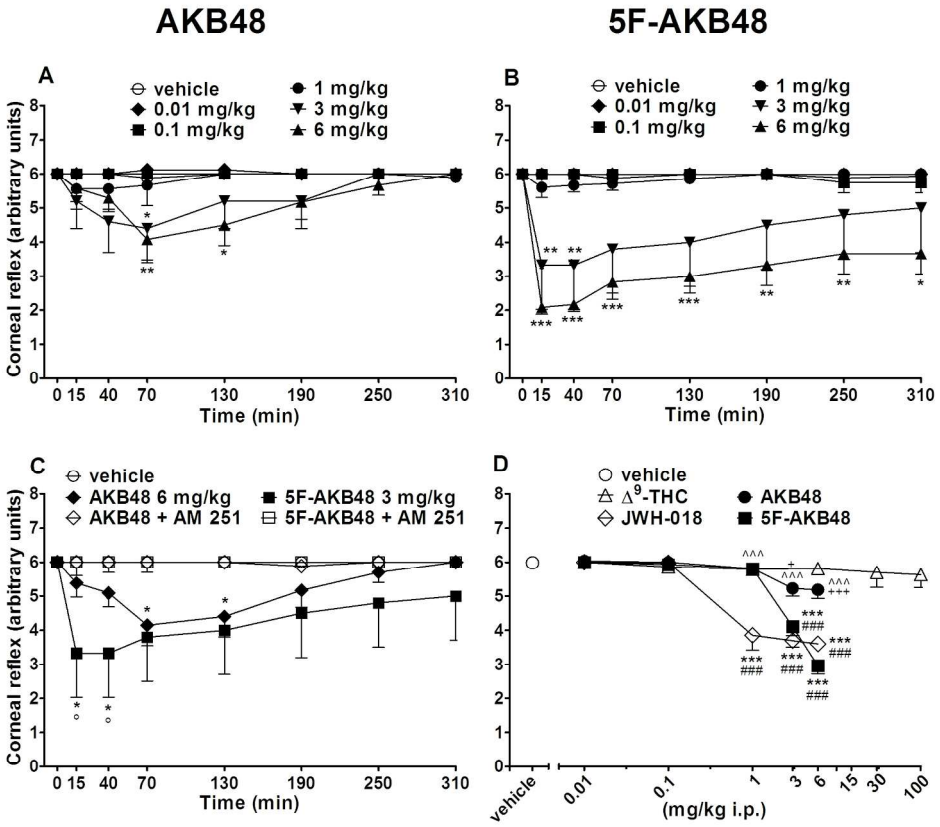

Figure 6 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the corneal reflex of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey\'s test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; ###p<0.001 versus Δ9-THC; ^^^p<0.001 versus JWH-018, +p<0.05, +++p<0.001 versus 5F-AKB48 and °p<0.05 versus AM 251+ agonist

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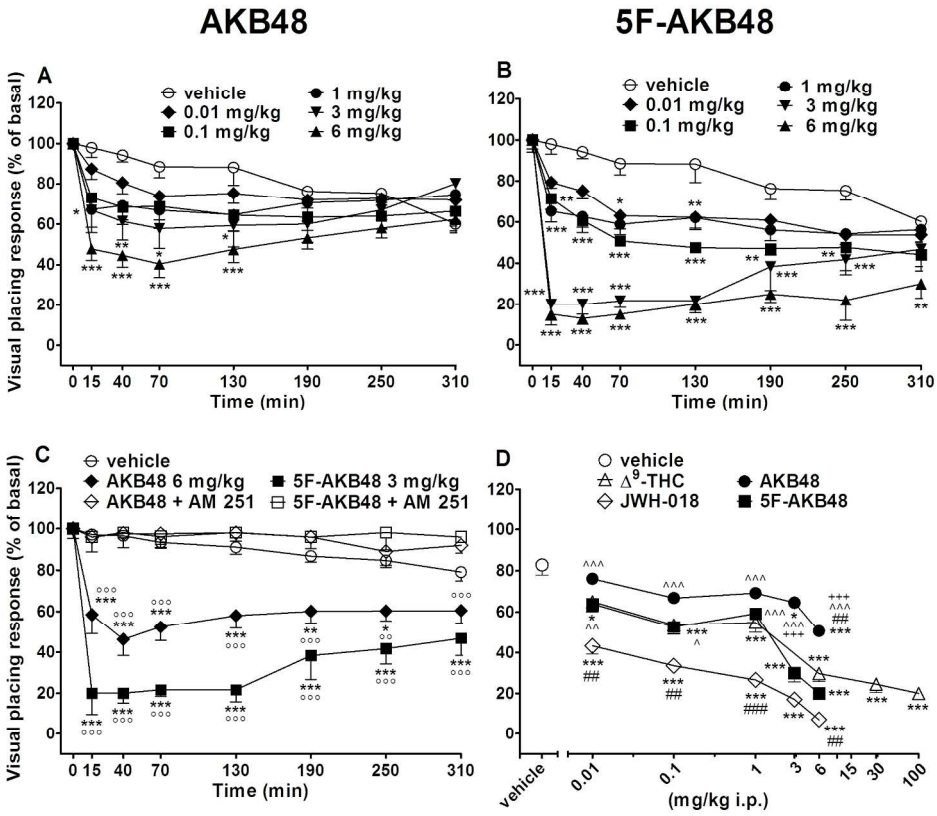

Figure 7 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the visual placing test of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey\'s test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; ##p<0.01, ###p<0.001 versus Δ9-THC; ^p<0.05, ^^p<0.01, ^^^p<0.001 versus JWH-018, +++p<0.001 versus 5F-AKB48 and °°p<0.01, °°°p<0.001 versus AM 251+ agonist
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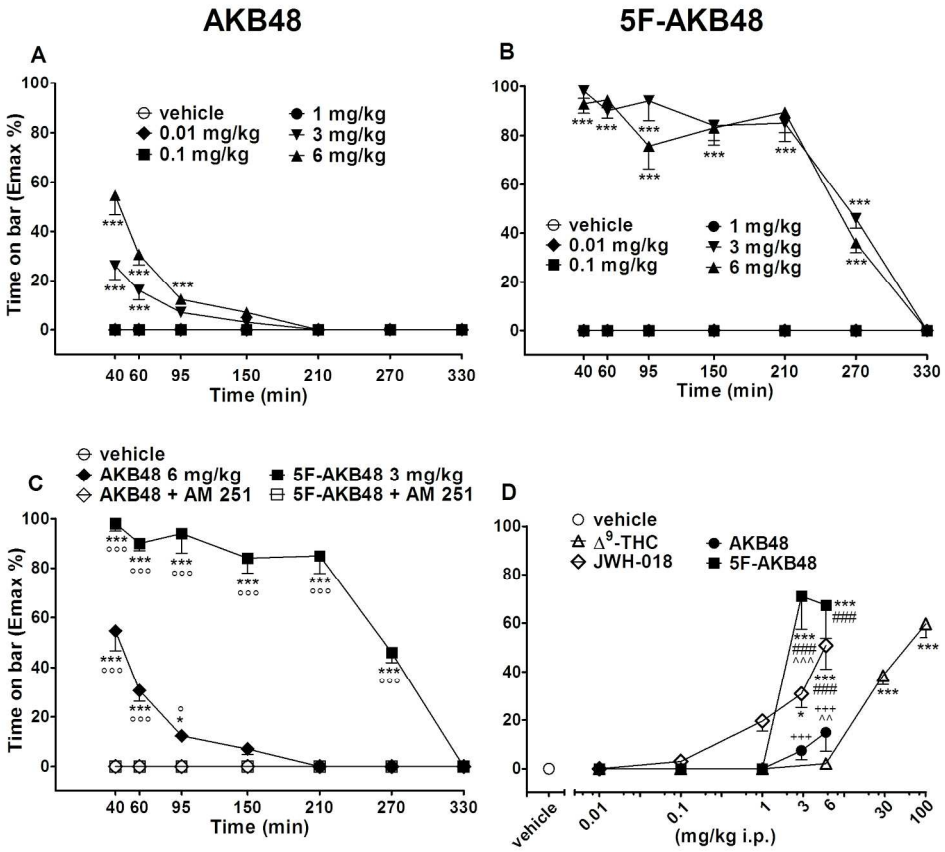

Figure 8 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the bar test of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey\'s test for multiple comparisons. *p<0.05, ***p<0.001 versus vehicle; ###p<0.001 versus Δ9-THC; ^^p<0.01, ^^^p<0.001 versus JWH-018, +++p<0.001 versus 5F-AKB48 and °p<0.05, °°°p<0.001 versus AM 251+ agonist

207x184mm (300 x 300 DPI)

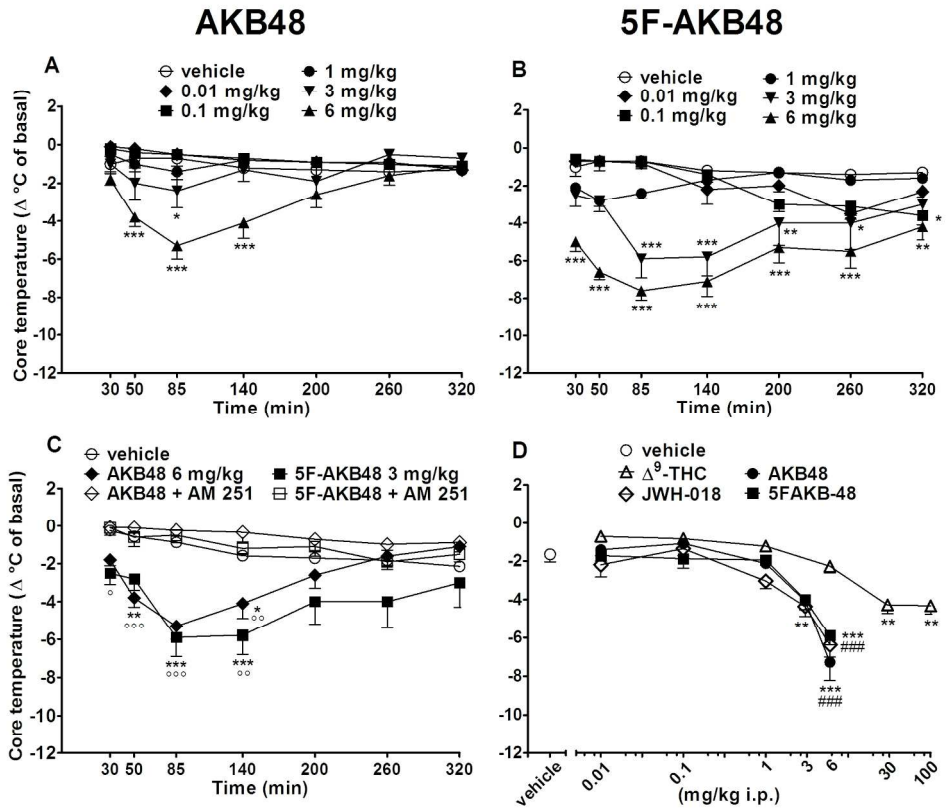

Figure 9 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on mouse core temperature. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey\'s test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; ###p<0.001 versus Δ9-THC; ^^^p<0.001 versus JWH-018, +++p<0.001 versus 5F-AKB48 and °p<0.05, °°p<0.01, °°°p<0.001 versus AM 251+ agonist

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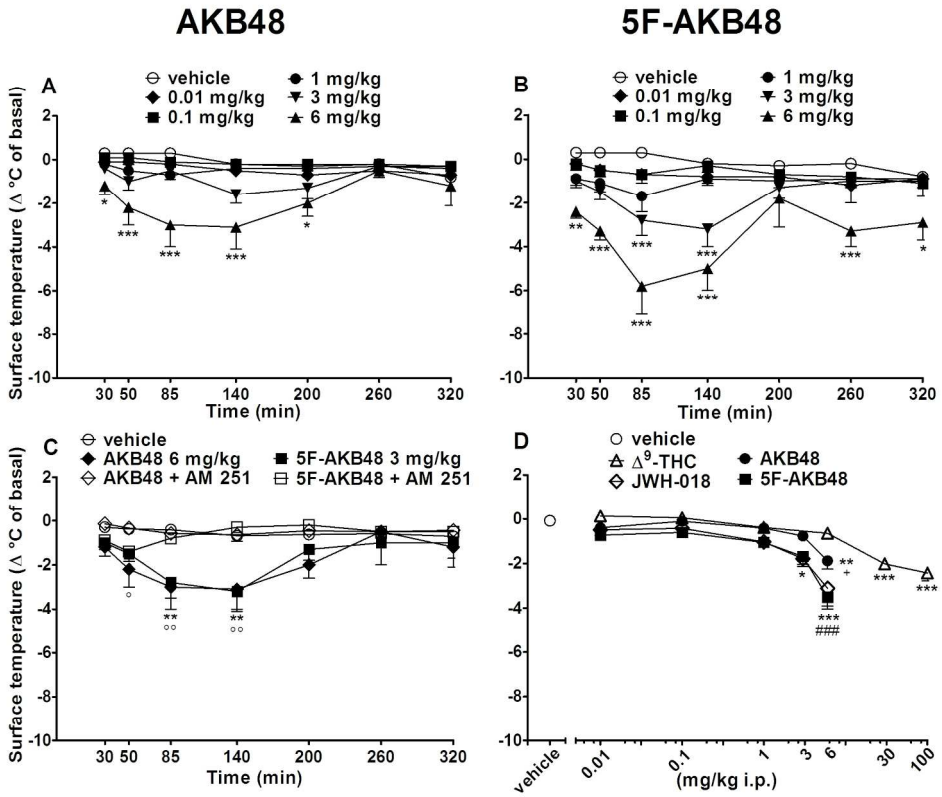

Figure 10 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the surface temperature of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey\'s test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; ###p<0.001 versus Δ9-THC; +p<0.05 versus 5F-AKB48 and °p<0.05, °°p<0.01 versus AM 251+ agonist
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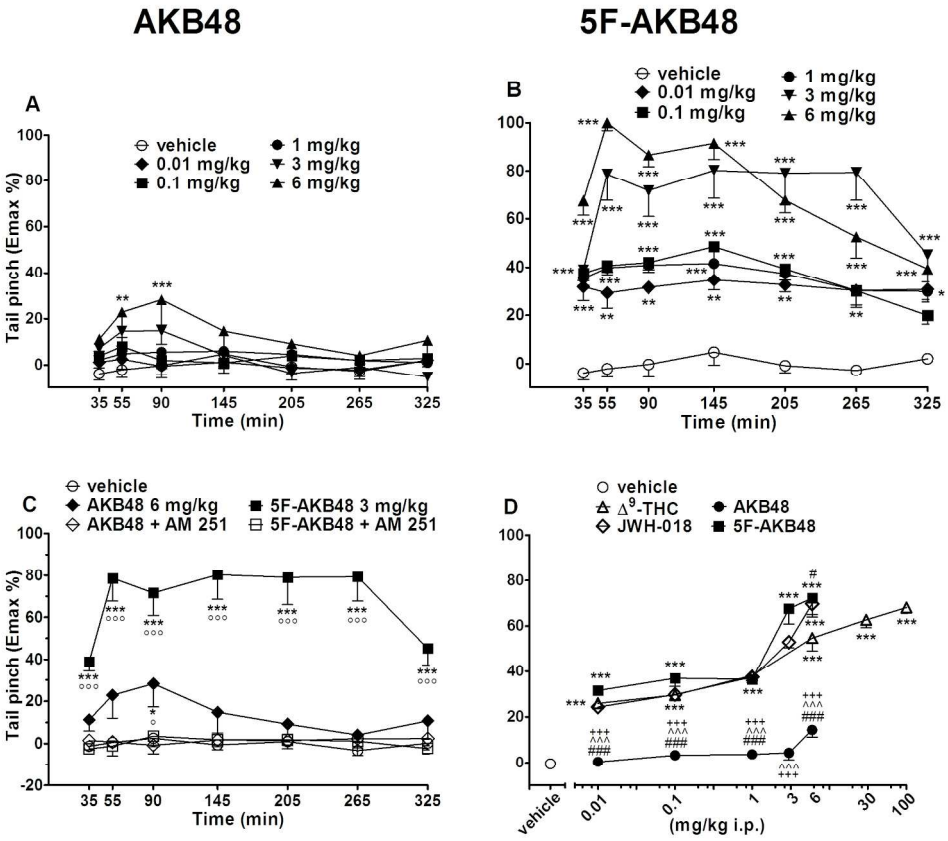

Figure 11 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the tail pinch test of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey\'s test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; #p<0.05, ###p<0.001 versus Δ9-THC; ^^^p<0.001 versus JWH-018, +++p<0.001 versus 5F-AKB48 and °°°p<0.001 versus AM 251+ agonist

208x183mm (300 x 300 DPI)

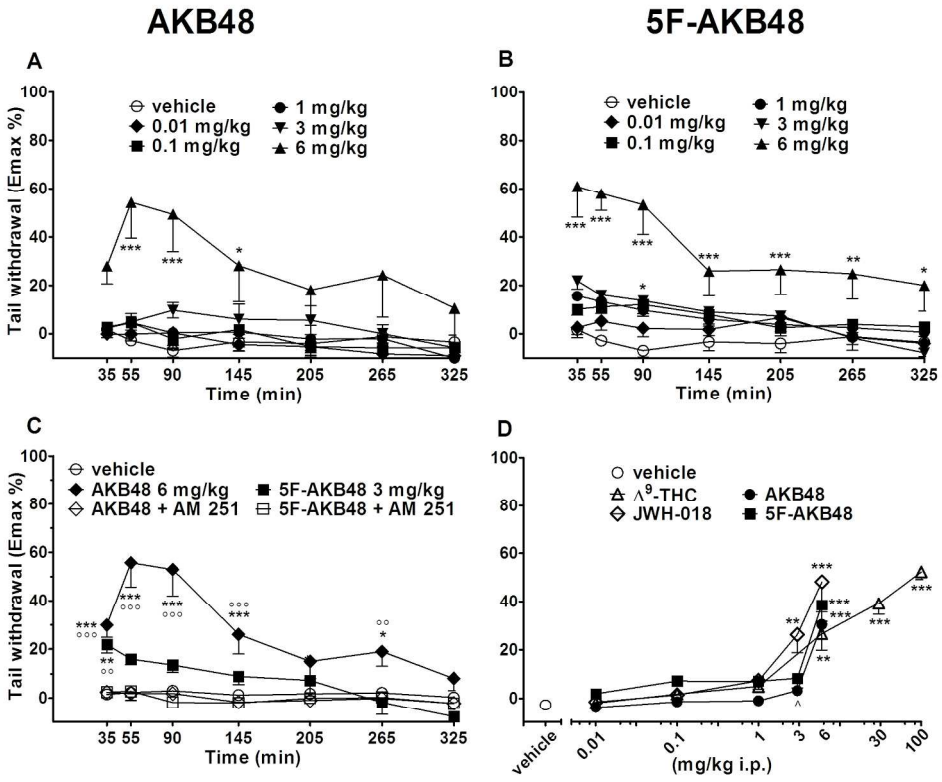

Figure 12 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the tail withdrawal test of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey\'s test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; ^p<0.05 versus JWH-018 and °°p<0.01, °°°p<0.001 versus AM 251+ agonist
209x172mm (300 x 300 DPI)

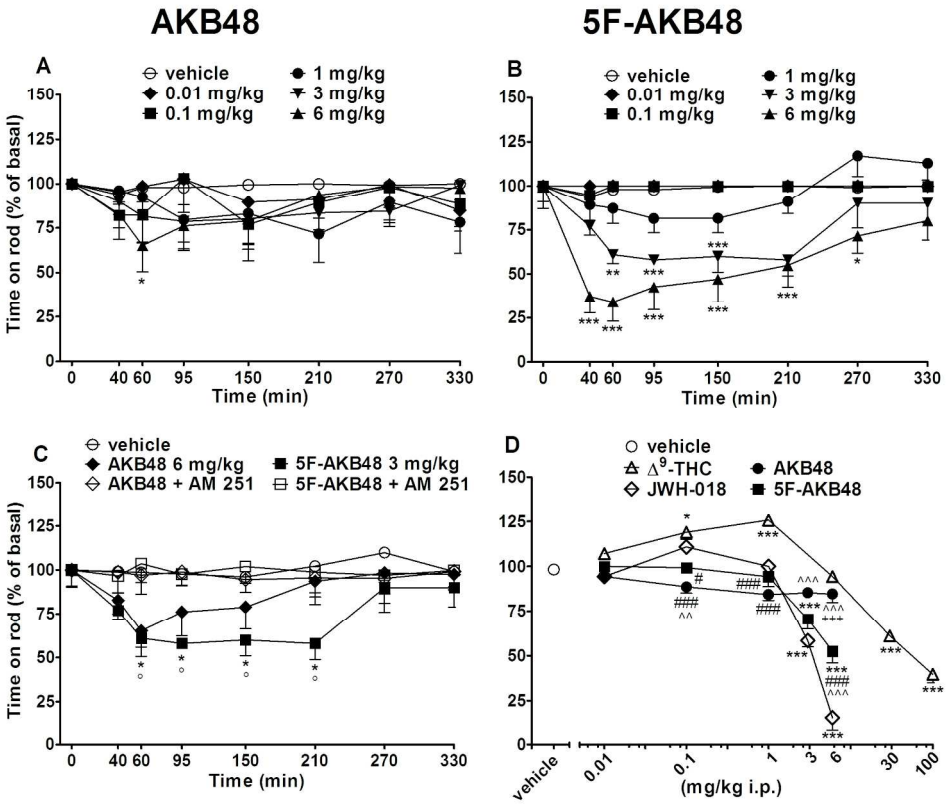

Figure 13 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the accelerod test of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey\'s test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; #p<0.05, ###p<0.001 versus Δ9-THC; ^^p<0.01, ^^^p<0.001 versus JWH-018, +++p<0.001 versus 5F-AKB48 and °p<0.05 versus AM 251+ agonist

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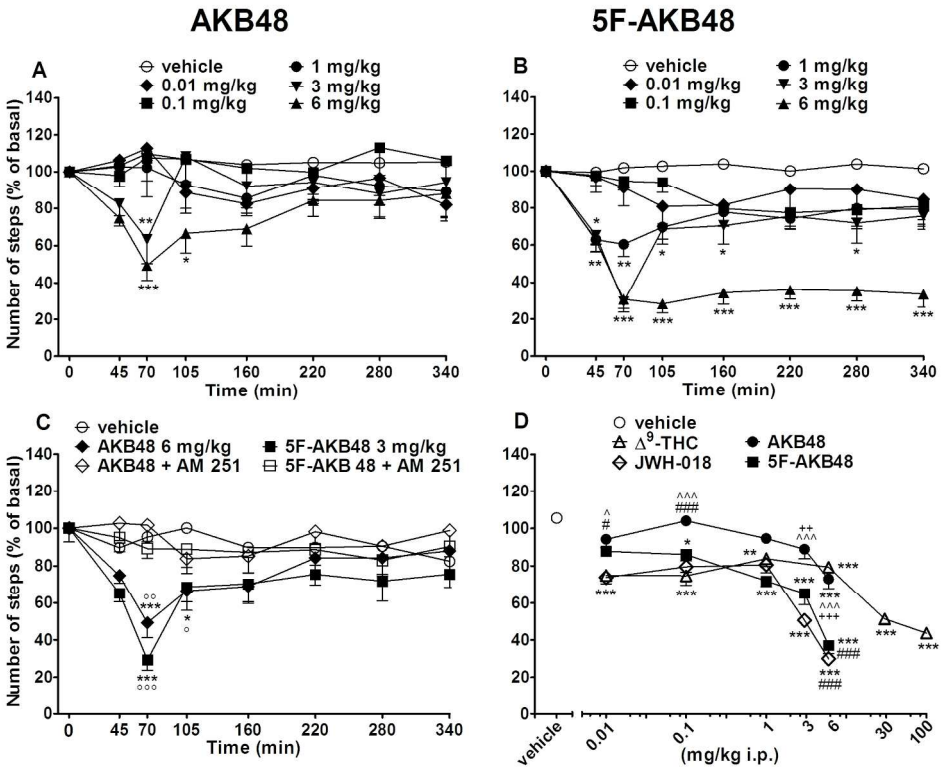

Figure 14 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A) and 5F AKB48 (panel B) on the drag test of mice. Interaction of effective dose of AKB48 and 5F AKB48 (6 mg/kg) with the selective CB1 receptor antagonist AM 251 (6 mg/kg, i.p.; panel C). Comparison of the total average effect observed in 5 hours (panel D) with Δ9-THC (0.01-100 mg/kg) and JWH-018 (0.01-6 mg/kg i.p.). Data are expressed (see material and methods) as arbitrary units and represent mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compound at different times (panel A, B) and for the interaction with the AM 251 (panel C), while the statistical analysis of the comparison of the total average effect of the compounds (panel D) was performed with one-way ANOVA followed by Tukey\'s test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001 versus vehicle; #p<0.05, ###p<0.001 versus Δ9-THC; ^p<0.05, ^^^p<0.001 versus JWH-018, ++p<0.01, +++p<0.001 versus 5F-AKB48 and °p<0.05, °°p<0.01, °°°p<0.001 versus AM 251+ agonist
208x168mm (300 x 300 DPI)

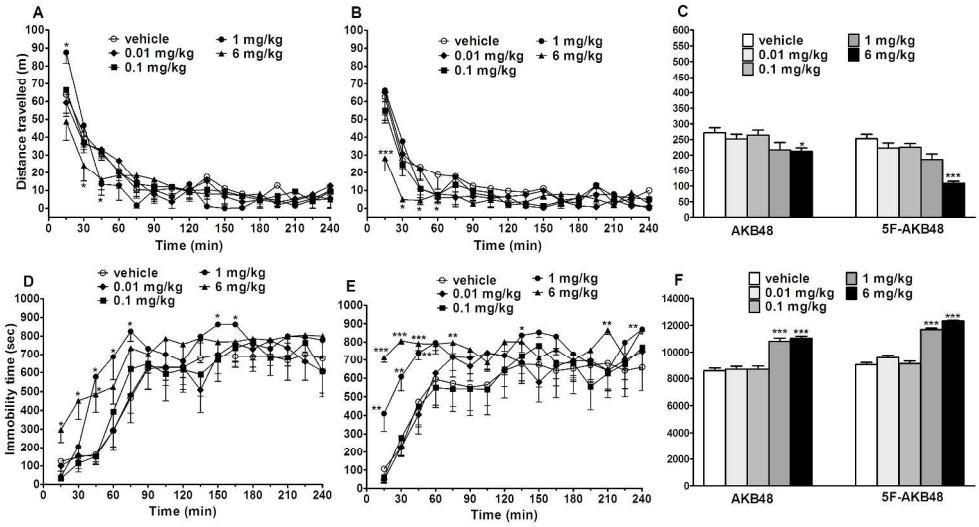

Figure 15 Effect of the systemic administration (0.01-6 mg/kg i.p.) of AKB48 (panel A and D) and Δ9-THC (panel B and E) on the total distance travelled and the total time immobile of the mouse. The overall effect observed in 5 hours (panel C and F) was also reported. Data are expressed as meters travelled (total distance travelled; panel A, B and C) and seconds of immobility (total time immobile; panel D, E and F) and represent the mean ± SEM of 10 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose response curve of AKB48 (panel A and D) and 5F-AKB48 (panel B and E) while the analysis of the overall effect (panel C and F) was performed with one-way ANOVA followed by Tukey\'s test for multiple comparisons. *p<0.05, ***p<0.001 versus vehicle
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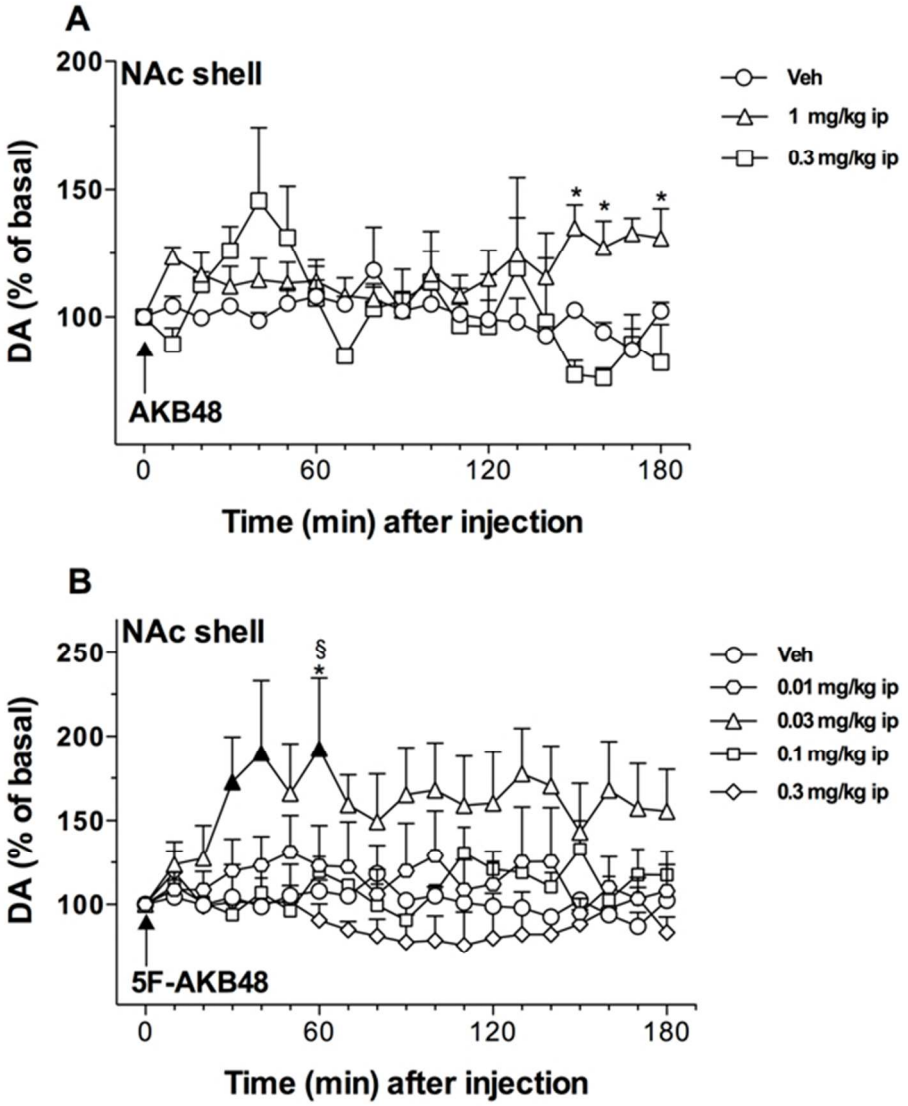

Figure 16 Effect of the systemic administration of AKB-48 (0.3, 1 mg/kg i.p.; panel A) and 5F-AKB48 (0.01-0.3 mg/kg i.p.; panel B) on DA transmission in the NAc shell of mice. Results are expressed as mean ± SEM of change in DA extracellular levels expressed as the percentage of basal values. Panel A: the arrow indicates the start of AKB-48 i.p. injection at the dose of 0.3 mg/kg (squares), 1 mg/kg (triangles), or vehicle (circles) in the NAc shell. Solid symbols: $p < 0.05$ with respect to basal values; *$p < 0.05$ vs 0.3 group; (NAc shell N=12) (Two-way ANOVA, Tukey\'s HSD post hoc). Panel B: the arrow indicates the start of 5F-AKB-48 i.p. injection at the dose of 0.01 mg/kg (circles), 0.03 mg/kg (triangles), 0.1 mg/kg (squares), 0.3 mg/kg (diamonds), or vehicle (circles) in the NAc shell. Solid symbol: $p < 0.05$ with respect to basal values; §$p < 0.05$ vs 0.3 group; *$p < 0.05$ vs veh; (NAc shell N=13) (Two-way ANOVA, Tukey\'s HSD post hoc)
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