

# Accepted Manuscript

Native CB1 receptor affinity, intrinsic activity and accumbens shell dopamine stimulant properties of third generation SPICE/K2 cannabinoids: BB-22, 5F-PB-22, 5F-AKB-48 and STS-135

Maria Antonietta De Luca, M. Paola Castelli, Barbara Loi, Alessandra Porcu, Mariella Martorelli, Cristina Miliano, Kathryn Kellett, Colin Davidson, L. Jacqueline Stair, Fabrizio Schifano, Gaetano Di Chiara

PII: S0028-3908(15)30181-7

DOI: [10.1016/j.neuropharm.2015.11.017](https://doi.org/10.1016/j.neuropharm.2015.11.017)

Reference: NP 6083

To appear in: *Neuropharmacology*

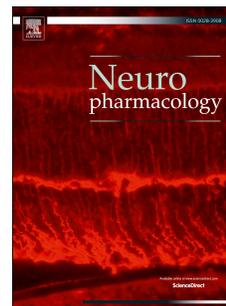
Received Date: 7 August 2015

Revised Date: 18 November 2015

Accepted Date: 19 November 2015

Please cite this article as: De Luca, M.A., Castelli, M.P., Loi, B., Porcu, A., Martorelli, M., Miliano, C., Kellett, K., Davidson, C., Stair, L.J., Schifano, F., Di Chiara, G., Native CB1 receptor affinity, intrinsic activity and accumbens shell dopamine stimulant properties of third generation SPICE/K2 cannabinoids: BB-22, 5F-PB-22, 5F-AKB-48 and STS-135, *Neuropharmacology* (2016), doi: 10.1016/j.neuropharm.2015.11.017.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



**NATIVE CB1 RECEPTOR AFFINITY, INTRINSIC ACTIVITY AND ACCUMBENS SHELL  
DOPAMINE STIMULANT PROPERTIES OF THIRD GENERATION SPICE/K2  
CANNABINOIDS: BB-22, 5F-PB-22, 5F-AKB-48 AND STS-135**

**Maria Antonietta De Luca<sup>1\*</sup>, M. Paola Castelli<sup>1\*</sup>, Barbara Loi<sup>2</sup>, Alessandra Porcu<sup>1</sup>, Mariella Martorelli<sup>1</sup>, Cristina Miliano<sup>1</sup>, Kathryn Kellett<sup>2</sup>, Colin Davidson<sup>3</sup>, L. Jacqueline Stair<sup>2</sup>, Fabrizio Schifano<sup>2</sup>, Gaetano Di Chiara<sup>1,4</sup>**

<sup>1</sup>Department of Biomedical Sciences, University of Cagliari, Italy

<sup>2</sup>School of Life and Medical Sciences, University of Hertfordshire, UK

<sup>3</sup>Pharmacology and Cell Physiology, Division of Biomedical Science, St George's University of London, London, UK

<sup>4</sup>CNR Institute of Neuroscience, Cagliari Section, University of Cagliari, Italy

\*Equal contribution

To whom correspondence should be addressed:

Dr. Maria Antonietta De Luca & Prof. Gaetano Di Chiara  
Department of Biomedical Sciences,  
University of Cagliari,  
Via Ospedale, 72  
09124 Cagliari, Italy  
Email: [deluca@unica.it](mailto:deluca@unica.it); [dichiara@unica.it](mailto:dichiara@unica.it)

**Abstract**

In order to investigate the *in vivo* dopamine (DA) stimulant properties of selected 3<sup>rd</sup> generation Spice/K2 cannabinoids, BB-22, 5F-PB-22, 5F-AKB-48 and STS-135, their *in vitro* affinity and agonist potency at native rat and mice CB1 receptors was studied. The compounds bind with high affinity to CB1 receptors in rat cerebral cortex homogenates and stimulate CB1-induced [<sup>35</sup>S]GTP $\gamma$ S binding with high potency and efficacy. BB-22 and 5F-PB-22 showed the lowest  $K_i$  of binding to CB1 receptors (0.11 and 0.13 nM), i.e., 30 and 26 times lower respectively than that of JWH-018 (3.38 nM), and a potency ( $EC_{50}$ , 2.9 and 3.7 nM, respectively) and efficacy ( $E_{max}$ , 217% and 203%, respectively) as CB1 agonists higher than JWH-018 ( $EC_{50}$ , 20.2 nM;  $E_{max}$ , 163%). 5F-AKB-48 and STS-135 had higher  $K_i$  for CB1 binding, higher  $EC_{50}$  and lower  $E_{max}$  as CB1 agonists than BB-22 and 5F-PB-22 but still comparatively more favourable than JWH-018.

The agonist properties of all the compounds were abolished or drastically reduced by the CB1 antagonist/inverse agonist AM251 (0.1  $\mu$ M). No activation of G-protein was observed in CB1-KO mice. BB-22 (0.003-0.01 mg/kg i.v.) increased dialysate DA in the accumbens shell but not in the core or in the medial prefrontal cortex, with a bell shaped dose-response curve and an effect at 0.01 mg/kg and a biphasic time-course. Systemic AM251 (1.0 mg/kg i.p.) completely prevented the stimulant effect of BB-22 on dialysate DA in the NAc shell. All the other compounds increased dialysate DA in the NAc shell at doses consistent with their *in vitro* affinity for CB1 receptors (5F-PB-22, 0.01 mg/kg; 5F-AKB-48, 0.1 mg/kg; STS-135, 0.15 mg/kg i.v.). 3<sup>rd</sup> generation cannabinoids can be even more potent and super-high CB1 receptor agonists compared to JWH-018. Future research will try to establish if these properties can explain the high toxicity and lethality associated with these compounds.

**Keywords:** cannabinoids, CB1 receptors, dopamine, microdialysis, Spice, K2, GTP $\gamma$ S binding

**Abbreviations**

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

**BB-22** 1-(cyclohexylmethyl)-1*H*-indole-3-carboxylic acid 8-quinolinyl ester

**5F-AKB-48** *N*-(adamantan-1-yl)-1-(5-fluoropentyl)-1*H*-indazole-3-carboxamide

**5F-PB-22** 1-pentylfluoro-1*H*-indole-3-carboxylic acid 8-quinolinyl ester

**DA** dopamine

**mPFC** medial Prefrontal Cortex

**NAc** Nucleus Accumbens

**NPS** new/novel psychoactive substance

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

**STS-135** *N*-(Adamantan-1-yl)-1-(5-fluoropentyl)-1*H*-indole-3-carboxamide

**THC** delta-9-tetrahydrocannabinol

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

## 1. INTRODUCTION

Herbal mixtures containing synthetic cannabinoid (SC) receptor agonists and intended to be used as a Marijuana surrogate, have been traded in Europe through the Internet under the name of Spice since 2004 and in North America, as K2, since 2008 (EMCDDA, 2009). Batches of the same brand may possess highly variable SC concentrations (Auwarter et al., 2013; Brents and Prather, 2014; Castaneto et al., 2015; Schifano et al., 2015; Schlatter, 2014; Zawilska and Wosjciechzak, 2013). Over the last few years, SC have gained popularity and especially so in adolescents and young adults. A recent US survey of SC use among students shows a prevalence of 7.4-7.9 % in those aged 15/18 years, with Spice products being the second most used drugs after Marijuana (Johnston et al., 2013).

SC identified in herbal mixtures can be classified into at least 7 categories: cyclohexylphenol (cannabicyclohexanol, CCH and CP-47497), classical cannabinoids (HU-210), naphthoylindoles (JWH-018 and JWH-073), phenylacetylindoles (JWH-250 and JWH-203), benzoylindoles (AM-694 and RCS-4), naphthoynaphthalenes (CB-13), adamantylindoles (APICA) and adamantylindazoles (AKB-48 "APINACA" and its 5-F derivative). SC intake has been associated with the occurrence of florid/acute transient psychosis, relapse/worsening of a pre-existing psychosis, persisting psychotic disorders/"spiceophrenia", and manic-like symptoms or relapse of pre-existing bipolar disorder (Papanti et al., 2013; Spaderna et al, 2013; van Amsterdam et al, 2015). A number of analytically confirmed accidental deaths/suicides have been related to SC ingestion, either on their own or in combination with other compounds (Brents and Prather, 2014; Papanti et al., 2014; Schifano et al., 2015).

On the basis of the temporal sequence of their appearance in Spice and K2 samples, three generations of SC can be distinguished (ACMD 2009, 2012, 2014). First generation Spice cannabinoids, JWH-018, CP 47,497 and HU-210 (ACMD, 2009), are full CB1 receptor agonists with affinities that are 4.5, 8.6 and 55 times that of THC respectively. Moreover, in functional tests

the above compounds act as full agonists, in contrast to the partial agonist activity of THC (Huffman et al., 2005; Atwood et al., 2010, 2011; Marshall et al., 2014).

JWH-018 has been recently shown to be self-administered i.v. by rats and mice, to increase extracellular dopamine (DA) in the shell of the nucleus accumbens (NAc) and to reduce GABA-A receptor inhibition of ventral tegmental area DA neurons in a cannabinoid receptor-dependent fashion (De Luca et al., 2015). These properties indicate that JWH-018 demonstrates positive reinforcing properties and resembles THC in its DA stimulant properties on DA transmission in the NAc (Tanda et al., 1997; Lecca et al., 2006; De Luca et al., 2012).

In 2009 various European countries and in 2010 various US States regulated the sale and use of cannabimimetic ingredients of Spice. This prompted a drastic reduction of the presence of 1<sup>st</sup> generation SC in Spice and K2-like preparations and their substitution with 2<sup>nd</sup> generation SC (ACMD, 2012). These compounds included haloalkyl derivatives of JWH-018, AM-2201 and its methyl derivative MAM-2201 and the fluoro alkyl, iodobenzyl derivative AM-694, the n-methylpiperidinyl AM-2233 and AM-1220, the benzoyl indoles AM-679, RCS-4 and derivatives, and adamantoindoles AM-1248 and AB-001. The last (e.g. the 3<sup>rd</sup>) generation of cannabinoids reported in herbal mixtures and in biological samples include compounds with an indazole or benzimidazole core replacing the indole (e.g. AKB-48, 5F-AKB-48, FUBIMINA), replacement of the carbonyl link of JWH-018 with carboxamide or carboxylate groups (e.g. APICA, SDB005), quinolinyl (PB-22 "QUPIC", 5F-PB-22, BB-22 "QUCHIC") or non-cyclic (ABDICA, AB-PINACA, 5F-AB-PINACA) secondary structures and novel nitrogenized tails (AB-FUBINACA, AB-FUBICA) (Uchiyama et al., 2012; 2013 a,b; ACMD, 2014).

To date, detailed information on the affinity, agonist potency, intrinsic activity and *in vivo* effects of these 3<sup>rd</sup> generation cannabinoids is lacking. As part of our continuing interest on the mechanism of the reinforcing properties and abuse liability of natural and synthetic cannabinoids, we selected four of the 3<sup>rd</sup> generation cannabinoids found in Spice/K2 products, namely BB-22, 5F-PB-22, 5F-AKB-48 (also known as 5F-APINACA) and STS-135. In the UK, synthetic

cannabinoids are the most frequently represented Novel Psychoactive Substance (NPS) category identified in samples submitted for analysis by a range of sources, with 5F-AKB-48 and 5-FPB-22 ('clockwork orange'; 'exodus'; and others) having been reported as the most identified NPS molecules overall (Wedinos, 2014). The recent trend of SC fluorination may increase the compounds' lipophilicity, hence enhancing the absorption through biological membranes/blood brain barrier (Schifano et al., 2015). The fluorinated SC STS-135 can be identified either on its own ('clockwork orange' and others) or in combination with other 'spice' molecules in products such as 'Moroccan' (Wedinos, 2014). Similarly, 5F-PB-22 ('Psyclone' and others) is the terminally fluorinated analogue of QUPIC (also known as PB-22). Psyclone incense is priced around £10 per gram, with users reporting intense and long-lasting euphoria/relaxation/visual hallucinations effects (Santacrose et al., 2015). Furthermore, BB-22, identified in products such as 'Vertex' and others, has recently been associated with a range of hospitalizations in the UK (Plymouth Herald, 2015). In this study, the above compounds' binding and agonist properties on native CB1 receptors in brain homogenates from rats and mice have been investigated. In addition, the *in vivo* effects on DA transmission, as monitored by brain microdialysis, of the compound which provided with the highest potency and efficacy as an agonist of CB1 receptors, namely BB-22, was studied in detail. Thus, the effect of BB-22 on DA transmission in the rat NAc shell and core and medial prefrontal cortex (mPFC) was investigated. The *in vivo* effects of the other three compounds on NAc shell DA transmission was also tested.

## 2. MATERIALS AND METHODS

### 2.1 Animals

Male Sprague-Dawley rats (Harlan Italy), C57BL/J6 and CB1 knockout (KO) mice (originally bred on C57BL/6J background were kindly donated by Dr Aron H. Lichtman, Department of Pharmacology and Toxicology, Virginia, Commonwealth, Virginia) were used for *in vitro* experiments (rats of 200-250g and mice of 17-20 g, respectively) and for *in vivo* microdialysis (rats of 275-300 g). Rats and mice were housed 4 and 10 per cage, respectively, in standard plastic cages

with wood chip bedding, at temperature of  $22 \pm 2$  °C and 60% humidity and under a 12 h light/dark cycle (lights on from 7.00 a.m.). Tap water and standard laboratory rodent chow (Mucedola, Settimo Milanese, Italy) were provided *ad libitum* in the homecage. All animal experiments were carried out in accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research according to Italian (D.L. 116/92 and 152/06) and European Council directives (609/86 and 63/2010) and in compliance with the approved animal policies by the Ethical Committee for Animal Experiments (CESA, University of Cagliari) and the Italian Ministry of Health. We made all efforts to minimize pain and suffering, and to reduce the number of animals used.

## 2.2 Drugs

5'-O-(3-[ $^{35}$ S]thiotriphosphate) ([ $^{35}$ S]GTP $\gamma$ S) (1250 Ci/mmol), [ $^3$ H]CP,55940 (131.8 Ci/mmol) ((-)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol) were purchased from Perkin-Elmer Life Sciences, Inc. (Boston, MA, USA). Guanosine 5' - diphosphate (GDP), and guanosine 5' -O-(3-thiotriphosphate) (GTP $\gamma$ S) were obtained from Sigma/RBI (St. Louis, MO, USA). CP55,940, WIN-55,212-2 (WIN) JWH-018 and AM 251 were purchased from Tocris (Bristol, UK). 5F-AKB-48, 5F-PB-22, BB-22, and STS-135 were purchased from an Internet source ([www.researchchemist.co.uk](http://www.researchchemist.co.uk)).

For biochemical experiments, drugs were dissolved in dimethyl sulfoxide (DMSO). The DMSO concentration used in the different assays never exceeded 0.1% (v/v) and had no effects on [ $^3$ H]CP-55,940 binding and [ $^{35}$ S]GTP $\gamma$ S binding assay. For *in vivo* microdialysis, drugs were solubilized in 2% EtOH, 2% Tween 80 and 96 % saline and administered intravenously (i.v.; 1 ml/kg) at different doses depending on the group of animals. BB-22: 0.003-0.1 mg/kg; 5F-PB-22: 0.01 mg/kg; 5F-AKB-48 0.1 mg/kg; STS-135 0.15 mg/kg. AM-251 was administered intraperitoneally (i.p.; 3 ml/kg) at the dose of 1 mg/kg.

## 2.3 Chemical Characterization of Cannabinoids Sourced from the Internet

To confirm their identity and purity, the substances were evaluated using gas chromatography mass spectrometry with electron ionisation (GC-EI-MS), 400 MHz nuclear magnetic resonance spectroscopy (NMR), and high performance liquid chromatography (HPLC). Reference standards of the four cannabinoids were purchased from Chiron (Norway) for comparison. GC-EI-MS was used for the initial identification where the fragmentation pattern of all four Internet products correlated to the cannabinoid claimed on the label, when compared to that of the reference standard as well as the SWG Drug library (Version 2.1). The identification was further confirmed using NMR where the number of peaks and splitting patterns were consistent with the cannabinoid chemical structures and in line with spectra produced by SWG Drug. HPLC was then used to evaluate the purity of the cannabinoid products where the purity of 5F-AKB-48, 5F-PB-22, BB-22, and STS-135 were determined to be  $93 \pm 1\%$ ,  $95.2 \pm 0.8\%$ ,  $90.6 \pm 0.6\%$ , and  $91 \pm 2\%$ , respectively. Full methods and data interpretation are available in the *Supplementary Information*.

## 2.4 In Vitro Experiments

**2.4.1 [<sup>3</sup>H]CP-55,940 Binding Assay.** Rats were sacrificed by decapitation. Brains were collected and cerebral cortices were rapidly dissected and placed on an ice-cold plate. After thawing, tissues were homogenated in 20 volumes (w/v) of ice-cold TME buffer (50 mM Tris-HCl, 1 mM EDTA, and 3 mM MgCl<sub>2</sub>, pH 7.4). The homogenates were centrifuged at 1000g for 10 min at 4 °C, and the resulting supernatants were centrifuged at 45000g for 30 min at 4 °C. Aliquots of membranes were frozen at -80 °C until the day of experiment. The Bradford protein assay was used for protein determination using bovine serum albumin (BSA) as a standard in accordance with the supplier protocol (Bio-Rad, Milan, Italy). [<sup>3</sup>H]CP-55,940 binding was carried out as previously described (Manera et al., 2006). Briefly, the membranes (40-50 µg of protein) were incubated for 1 h at 30 °C with [<sup>3</sup>H]CP-55,940 (0.5 nM) in a final volume of 0.5 mL of TME buffer containing 5 mg/mL BSA. Nonspecific binding was determined in the presence of 10 µM CP-55,940. Incubation was terminated by rapid filtration through Whatman GF/C filters pretreated with 0.5% (w/v) polyethyleneimine (PEI), using a Brandell 30-sample harvester (Gaithersburg, MD). Filters were

washed three times with ice-cold Tris-HCl buffer (pH 7.4) containing 1 mg/ml BSA. Filter-bound radioactivity was counted in a liquid scintillation counter (Packard Tricarb 2810 TR, Packard, Meridien, CT), using 3 mL of scintillation fluid (Ultima Gold Packard, MV, Meridien, CT). [<sup>3</sup>H]CP-55,940 displacement curves were plotted using serial dilutions ranging from 10<sup>-11</sup> to 10<sup>-5</sup> M unlabeled compounds and [<sup>3</sup>H]CP-55,940 (0.5 nM). Independent experiments were repeated on membrane preparations from at least three different experiments. The calculation of the IC<sub>50</sub> (the concentration that inhibits 50% of specific radioligand binding) was performed by nonlinear curve fitting of the concentration-effect curves using GraphPad Prism, San Diego, CA. The *F*-test was used to determine the best approximation of a nonlinear curve fit to a one- or two- site model (*P* < 0.05). IC<sub>50</sub> values were converted to *K*<sub>i</sub> values by means of the Cheng and Prusoff equation (Cheng and Prusoff, 1973).

**2.4.2 [<sup>35</sup>S]GTPγS Binding Assay.** Rat and mouse cortical membranes were suspended in 20 volumes of cold centrifugation buffer (50 mM Tris-HCl, 3 mM MgCl<sub>2</sub>, 1 mM EDTA, pH 7.4) and homogenized using a homogenizer system (Glass-Col, Terre Haute, IN). The homogenate was centrifuged at 48000g for 10 min at 4 °C. The pellet was then resuspended in the same buffer, homogenized, and centrifuged as previously described. The final pellet was subsequently resuspended in assay buffer (50 mM Tris-HCl, 3 mM MgCl<sub>2</sub>, 0.2 mM EGTA, 100 mM NaCl, pH 7.4), homogenized, and diluted to a concentration of ~2 mg/mL with assay buffer. Membrane aliquots were then stored at -80 °C until use. [<sup>35</sup>S]GTPγS binding was measured as previously described (Manera et al., 2006). Briefly, mouse and rat brain membranes (5-10 μg of protein) were incubated with compounds at 30 °C in assay buffer containing 0.1% BSA in the presence of 0.05 nM [<sup>35</sup>S]GTPγS and 30 μM GDP in a final volume of 1 ml. After 60 min incubation, samples were filtered using a Packard Unifilter-GF/B, washed twice with 1 ml of ice-cold 50 mM Tris-HCl, pH 7.4 buffer, and dried for 1 h at 30 °C. The radioactivity on the filters was counted in a liquid microplate scintillation counter (TopCount NXT, Packard, Meridien, CT) using 30 μl of scintillation fluid (Microscint 20, Packard, Meridien, CT). Concentration-effect curves were

determined by incubating membranes with various concentrations of compounds (0.1 nM-10  $\mu$ M) in the presence of 0.05 nM [<sup>35</sup>S]GTP $\gamma$ S and 30  $\mu$ M GDP. Nonspecific binding was measured in the presence of 10  $\mu$ M unlabeled GTP $\gamma$ S. Basal binding was assayed in the absence of agonist and in the presence of GDP. Stimulation by the agonist was defined as a percentage increase above basal levels (i.e.,  $\{[\text{dpm}(\text{agonist}) - \text{dpm}(\text{no agonist})]/\text{dpm}(\text{no agonist})\} \times 100$ ). Nonlinear regression analysis of concentration-response data was performed using Prism 6.0 software (GraphPad Prism program, San Diego, CA) to calculate  $E_{\text{max}}$  (maximal stimulation over basal levels) and  $EC_{50}$  (concentration of agonist to obtain 50% of the maximal effect) values.

## 2.5 *In vivo* microdialysis

**2.5.1 Surgery.** Male Sprague-Dawley rats (275-300 g; Harlan, Italy) were anaesthetised with Equitesin (3ml/kg ip; chloral hydrate 2.1 g, sodium pentobarbital 0.46 g, MgSO<sub>4</sub> 1.06 g, propylene glycol 21.4 ml, ethanol (90%) 5.7 ml, H<sub>2</sub>O 3 ml) placed in a stereotaxic apparatus and implanted with vertical dialysis probes (1.5 or 3 mm dialyzing portion for NAc or mPFC, respectively) in the NAc shell (A+2.2, L+1.0 from bregma, V-7.8 from dura) or core (A+1.4; L+1.6 from bregma; V-7.6 from dura) or in the mPFC (A+3.7, L+0.8 from bregma, V-5.0 from dura), according to the rat brain atlas of Paxinos and Watson (1998). In order to perform intravenous (i.v.) drug administration, a catheter (Silastic, Dow Corning Corporation, Michigan, USA) was inserted in the right jugular vein as previously described (De Luca et al., 2014).

**2.5.2 Analytical Procedure.** On the day following surgery, probes were perfused with Ringer's solution (147 mM NaCl, 4 mM KCl, 2.2 mM CaCl<sub>2</sub>) at a constant rate of 1  $\mu$ l/min. Dialysate samples (10  $\mu$ l) were injected into an HPLC equipped with a reverse phase column (C8 3.5  $\mu$ m, 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<sub>2</sub>PO<sub>4</sub>, 0.1 mM Na<sub>2</sub>-EDTA, 0.5 mM n-octyl sodium sulfate, 15% (v/v) methanol, pH 5.5. The sensitivity of the assay for DA was 5 fmol/sample.

**2.5.3 Histology.** At the end of the experiment, animals were sacrificed and their brains removed and stored in formalin (8%) for histological examination to verify the correct placement of the microdialysis probe.

## 2.6 Statistical Analysis

All the numerical data are given as mean  $\pm$  SEM. Data were analyzed by utilizing one-way ANOVA or repeated measures ANOVA or T-test. Results from treatments showing significant overall changes were subjected to Tukey's tests or Dunnett's tests for *post hoc* comparisons, with significance for  $p < 0.05$ .

## 3. RESULTS

### 3.1 *IN VITRO* STUDIES

#### 3.1.1 Agonist-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding

As shown in Fig. 2 A-B, at 1  $\mu$ M concentration WIN and JWH-018, our reference compounds, stimulated [<sup>35</sup>S]GTP $\gamma$ S binding to rat cortex membranes to approximately 150% and 170%, respectively, of the basal activity. BB-22, 5F-PB-22, 5F-AKB-48, and STS-135 produced greater G-protein stimulation than the full CB1 receptor agonist, WIN. Specifically, the stimulation of GTP $\gamma$ S induced by 1  $\mu$ M of BB-22 and 5F-PB-22 was significantly ( $p < 0.01$ ) greater than the amount of stimulation produced by WIN (Fig. 1A). WIN and all compounds produced no GTP $\gamma$ S stimulation when co-incubated with AM 251 (0.1  $\mu$ M), a CB1 receptor antagonist/inverse agonist (Fig. 2 A-B), suggesting that all four test compounds activate a G protein coupled to the CB1 receptor.

[<sup>35</sup>S]GTP $\gamma$ S binding was stimulated in a concentration-dependent and saturable manner by the prototypic indole-derived synthetic cannabinoid JWH-018 and by all four synthetic cannabinoids 5F-AKB-48, STS-135, BB-22 and 5F-PB-22 (Fig. 3, Table 1). All compounds possess nanomolar potency at CB1 receptors, with BB-22 and 5F-PB-22 being approximately 5-7 fold more potent than JWH-018. EC<sub>50</sub> values for BB-22 and 5F-PB-22 were significantly lower than EC<sub>50</sub> value for JWH-018 (ANOVA:  $F_{(4,14)} = 14.78$ ,  $P < 0.0001$ ,  $p < 0.05$ , Dunnett's test), while no difference was

recorded in the  $EC_{50}$  value for STS-135 and 5F-AKB-48 (ANOVA:  $F_{(4,14)} = 14.78$   $P < 0.001$ ). These latter compounds display similar potency to JWH-018 for stimulating GTP $\gamma$ S binding-CB1 mediated (Table 1). The maximal efficacy ( $E_{max}$ ) of G-protein activation by JWH-018 and STS-135 was similar while the other compounds (5F-AKB-48, BB-22 and 5F-PB-22) exhibited significant enhanced efficacy compared to JWH-018 (ANOVA  $F_{(4,14)} = 11.56$   $P < 0.001$ ). The rank order of potency and efficacy was BB-22 = 5FP-22 > JWH-018 = 5F-AKB-48 = STS-135 and BB-22 = 5FP-22 > 5F-AKB-48 > STS-135 = JWH-018, respectively (Table 1). Lastly, to confirm the involvement of the cannabinoid CB1 receptor in the activation of G protein we performed concentration-effect curves of our compounds in mouse cortex membrane homogenates of CB1-KO and wild-type mice. As shown in Fig. 4, all compounds stimulated [ $^{35}$ S]GTP $\gamma$ S binding in a concentration-dependent manner in cortex of wild-type mice with  $EC_{50}$  and  $E_{max}$  values of  $38 \pm 5.7$  nM and  $158 \pm 2.4$  %,  $28 \pm 3.2$  nM and  $167 \pm 3.7$  %,  $15 \pm 1.7$  nM and  $159 \pm 1.5$  %,  $4 \pm 0.9$  nM and  $183 \pm 5.5$  %,  $1.46 \pm 0.14$  nM and  $187 \pm 3.6$  %, for JWH-018, 5F-AKB-48, STS-135, 5F-PB-22 and BB-22, respectively. Importantly, no activation of G protein was observed in CB1-KO mice.

### 3.1.2 Effects of JWH-018, 5F-AKB48, STS-135, BB-22 and 5F-PB-22 on cannabinoid CB1 receptor binding

To determine the affinity of JWH-018 and the other compounds to the CB1 receptor, we used a radiolabelled competition binding assay in rat cortical membranes. Indeed, high levels of CB1 receptors are expressed in the central nervous system, while only negligible CB2 receptors quantities are present (Pertwee, 2005). In good agreement with previous published data (Devane et al., 1988; Thomas et al., 1998), the  $K_d$  and  $B_{max}$  obtained by Scatchard analysis of [ $^3$ H]CP55,940 saturation binding were  $2.08 \pm 0.16$  picomol/mg protein and  $0.33 \pm 0.06$  nM, respectively ( $n=3$ , data not shown). As expected, JWH-018 in rat cortical membranes caused complete inhibition of the specific binding of [ $^3$ H]CP55,940 with a  $K_i$  of  $3.4 \pm 0.6$  nM (Fig. 4). As shown in Table 1, all four test compounds displaced [ $^3$ H]CP55,940 binding with varying affinities ranging from  $0.11 \pm 0.03$  for BB-22 to  $1.9 \pm 0.18$  for STS-135. Indeed,  $K_i$  values of these compounds were significantly

lower compared to our reference compound JWH-018, with the rank order of CB1 receptor affinity BB-22 = 5FPB-22 > 5F-AKB-48 > STS-135 > JWH-018 (Table 1).

### 3.2 *IN VIVO* MICRODIALYSIS STUDIES

Rat basal values of DA, expressed as fmoles/10  $\mu$ l sample (mean  $\pm$  SEM), were: NAc shell  $52 \pm 5$  (N =50), NAc core  $55 \pm 4$  (N =25), mPFC  $16 \pm 2$  (N =21).

#### 3.2.1 Effect of BB-22 administration on DA transmission in the NAc shell and core, and in the mPFC

In this first experiment, we studied the effect of four doses of BB-22 (0.003, 0.01, 0.03, 0.1, mg/kg i.v.) on extracellular DA levels in NAc shell and core, and mPFC. As shown in Fig. 6, the dose-response curve of the effect of BB-22 on dialysate DA is bell-shaped with the dose of 0.01 mg/kg increasing DA levels preferentially in the NAc shell as compared to the NAc core and mPFC. No significant effects were observed in the NAc core and mPFC. Three-way ANOVA showed a main effect of dose [ $F_{(3,75)}=4.46$ ;  $p < 0.01$ ], brain area [ $F_{(2,75)}=7.72$ ;  $p < 0.001$ ] and time [ $F_{(12,900)}=4.24$ ;  $p < 0.001$ ], and a significant dose x brain area interaction [ $F_{(6,75)}=6.46$ ;  $p < 0.0001$ ]. Tukey *post hoc* tests showed an increase of dialysate DA in the NAc shell after 0.01 mg/kg of BB-22 revealing differences at the 20-40 and 90-120 min sample with respect to basal value, to vehicle treated animals implanted in NAc shell, and to the same dose (0.01 mg/kg) treated animals implanted in the NAc core (90 min sample) and in the mPFC (30, 90 min sample).

#### 3.2.2 Role of CB1 receptors on the NAc shell DA stimulation induced by BB-22

In this experiment, we studied the effect of CB1 receptor blockade by the inverse agonists/antagonists AM 251 on the NAc shell DA response to BB-22 (0.01 mg/kg i.v.) in rats (Fig. 7). In AM 251 pre-treated animals, two-way ANOVA showed a main effect of treatment [ $F_{(1,11)}=12.07$ ;  $p < 0.005$ ], and treatment x time interaction [ $F_{(18,198)}=2.2$ ;  $p < 0.005$ ]. Tukey's *post*

*hoc* tests revealed that pre-treatment with AM-251 significantly reduced dialysate DA in the NAc shell as compared to rats pre-treated with vehicle (90, 140, 150, 180 min sample).

### 3.2.3 Effect of 5F-PB-22, 5F-AKB-48, and STS-135 administration on DA transmission in the NAc shell

In this set of experiments, we studied the effect of 5F-PB-22, 5F-AKB-48, and STS-135 on extracellular DA levels in the NAc shell. As shown in Fig. 8, all the drugs tested stimulated DA transmission in the NAc shell. A two-way ANOVA showed the following main effects: 5F-PB-22 treatment [ $F_{(1,10)}=15.97$ ;  $p < 0.005$ ]; 5F-AKB-48 treatment [ $F_{(1,11)}=63.39$ ;  $p < 0.001$ ], 5F-AKB-48 time x treatment [ $F_{(18,198)}=1.7$ ;  $p < 0.05$ ]; STS-135 time [ $F_{(18,144)}=2.16$ ;  $p < 0.05$ ], STS-135 time x treatment [ $F_{(18,144)}=2.1$ ;  $p < 0.005$ ]. Tukey *post hoc* tests showed a larger increase of dialysate DA in the NAc shell after all the cannabinoids tested revealing differences at the 30 and 40 min samples with respect to basal value (5F-PB-22); at the 60, 100 and 150 min samples with respect to basal value and at the 60 and 100 min samples compared to vehicle (5F-AKB-48); at the 60 min sample with respect to basal value and to vehicle (STS-135).

## 4. DISCUSSION

The main findings of this study are that the four 3<sup>rd</sup> generation cannabinoids, BB-22, 5F-PB-22, 5F-AKB-48 and STS-135 are high affinity ligands and potent full agonists at the native rat and mice brain CB1 receptors and stimulate *in vivo* DA transmission in the NAc shell at doses consistent with their *in vitro* CB1 receptor affinity. Our *in vitro* findings demonstrate that BB-22, 5F-PB-22, 5F-AKB-48 and STS-135 bind with nanomolar affinity to CB1 receptors in rat cerebral cortex homogenates and stimulate CB1-induced [ $^{35}$ S]GTP $\gamma$ S binding with high potency and efficacy. Previous research has shown that JWH-018, our reference compound, binds with and activates CB1 receptors with high affinity and potency and displays *in vivo* cannabimimetic activity, i.e., antinociception, hypothermia, catalepsy and locomotor suppression (Brents et al., 2011; Wiley et

al., 2012; Vigolo et al., 2015). The G protein activation induced by JWH-018 and by all compounds was completely suppressed by the CB1 antagonist/inverse agonist AM 251 and totally absent in CB1-KO mice. Taken together these data indicate that BB-22, 5F-PB-22, 5F-AKB-48 and STS-135 activate a G-protein coupled CB1 receptor. The rank order of potency and efficacy as CB1 receptor agonists correlated with CB1 receptor binding affinity, and all four compounds were full agonists in [<sup>35</sup>S]GTPγS binding studies as compared to JWH-018. Notably, the most potent compounds, the fluorinate 5F-PB-22 and BB-22, possess greater CB1 receptor agonist potency (5 and 7 fold, respectively) and efficacy and a higher binding affinity (26 and 30 fold, respectively) at CB1 receptors compared to JWH-018. Among the 4 SC, BB-22, the compound provided with the highest affinity for CB1 receptors and the highest intrinsic activity as a CB1 receptor agonist, was selected to be tested for its *in vivo* effects on DA transmission, as estimated by DA microdialysis. We examined BB-22 in three terminal DA areas, namely the NAc shell and core and the medial PFC. Using a range of 4 doses (0.003-0.1 mg/kg i.v.), BB-22 increased dialysate DA in the NAc shell at the intermediate dose of 0.01 mg/kg i.v. Most importantly, this effect was prevented by AM 251 at a dose that did not affect basal dialysate DA. The dose of BB-22 that increased dialysate DA in the NAc shell is about 10 times lower than the dose of JWH-018 that elicits a quantitatively similar peak increase of dialysate DA in the NAc shell (about 50% over basal) and compares favourably with the difference in  $K_i$  between the two compounds as ligands of native rat CB1 receptors (BB-22, 0.11 nM; JWH-018, 3.38 nM). BB-22 also shares with JWH-018 the bell shaped dose-response curve of the increase in NAc shell DA, with loss of the effect at the highest dose tested. Interestingly, the stimulation of dialysate DA in the NAc shell took place within a rather narrow range of doses, a feature that differentiates these compounds from THC as well as from psychostimulants, nicotine and narcotic drugs of abuse (Di Chiara et al, 2004; Pontieri et al., 1995 and 1996; Tanda et al., 1997). The other three compounds were tested for their effects on dialysate DA in the NAc shell at a single dose level, selected on the basis of the ratio between the  $K_i$  of JWH-018 and BB-22 for CB1 receptors and the doses of the same compounds that activate *in vivo*

NAc shell DA transmission. Thus, doses of 0.01 mg/kg i.v. of 5F-PB-22, 0.1 mg/kg of 5F-AKB-48 and 0.15 mg/kg of STS-135 were tested. At these doses, all compounds increased dialysate DA in the NAc shell to a similar extent to BB-22 (max < 50% over basal). In the case of 5F-AKB-48 the increase of dialysate DA was delayed. In the case of BB-22 the biphasic time-course of dialysate DA in the NAc shell could be due to the formation of active metabolites while the delayed effect of 5F-AKB-48 might be due to slow passage through the blood-brain-barrier due to steric hindrance related to the bulky adamantyl residue.

In conclusion, we have shown that four representatives of 3<sup>rd</sup> generation Spice/K2 cannabinoids are highly potent and effective agonists of CB1 receptors and, consistent with the properties of THC and synthetic cannabinoids agonists like WIN 55,212-2 (Lecca et al., 2006) and JWH-018 (De Luca et al., 2015), activate DA transmission selectively in the rat NAc shell. In the absence of direct evidence for reinforcing properties as deduced from i.v. self-administration studies, the property of activating DA transmission in the NAc shell provides circumstantial pre-clinical evidence for a putative abuse liability of these compounds. The present findings are a reason for further clinical concern. Users do not seem to be aware of the serious adverse effects related to SC misuse, since these compounds may be perceived to be somehow equivalent to Marijuana and hence “safe” and “natural” (Santacroce et al., 2015; Schifano et al., 2015). Appropriate, non-judgemental, prevention campaigns with a special focus on the differences between SC and cannabis may need to be organized on a large scale. At the same time, clinicians need to be regularly updated about NPS, including SC, to promptly recognize signs/symptoms of intoxication (Simonato et al., 2013; Papanti et al., 2014; Schifano et al., 2015).

**Figure legends****Figure 1****Structures of selected synthetic cannabinoids.****Figure 2****Effect of WIN, JWH-018 and its derivatives on [<sup>35</sup>S]GTP $\gamma$ S binding in rat cortical membranes.**

WIN, JWH-018, BB-22, 5F-PB-22 (2A), 5F-AKB-48 and STS-135 (2B) were tested alone or in combination with the CB1 receptor antagonist/inverse agonist, AM 251 (0.1  $\mu$ M). Data, expressed as percentage of basal values, are means  $\pm$  SEM of at least three determinations in triplicate. The horizontal dotted line indicates baseline values. One-way ANOVA: 2A,  $F_{(9,39)} = 42,45$   $P < 0.0001$ ; 2B:  $F_{(9,39)} = 37,30$   $P < 0.0001$  \*\* $p < 0.01$  vs WIN, Tukey' test.

**Figure 3****Concentration-response curves of compound-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding in rat cortical membranes.**

Data are expressed as mean percentage of basal values of GTP $\gamma$ S binding  $\pm$  SEM of at least four independent experiments. Rat cortical membranes were incubated with various concentrations of BB-22 (*black squares*), 5F-PB-22 (*red triangles*), 5F-AKB-48 (*green triangles*), STS-135 (*magenta diamonds*), and JWH-018 (*blue circles*), as described in Material and Methods. The parameters describing the different curves are given in Table 1.

**Figure 4****Concentration-response curves of compounds-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding in mouse cortical membranes of CB1-KO and wild-type mice.**

Data represent a typical experiment out of three independent experiments. EC<sub>50</sub> of Wild-Type mice: BB-22 (*black squares*), 1.7 nM; 5F-PB-22 (*red triangles*), 3.4 nM; 5F-AKB-48 (*green triangles*),

28 nM; STS-135 (*magenta diamonds*), 15 nM; JWH-018 (*blue circles*): 36 nM. All compounds fail to activate GTPγS binding in CB1-KO mice (*dotted lines*).

### Figure 5

**Displacement curves of [<sup>3</sup>H]CP55,940 in rat cortical membranes by BB-22, 5F-PB-22, 5F-AKB-48, STS-135, and JWH-018.**

Data are expressed as means ± SEM of at least four independent experiments, each performed in triplicate. The calculation of IC<sub>50</sub> was performed by non-linear curve fitting of the concentration-effect curves using GraphPad Prism. The F-test was used to determine the best approximation of a non-linear curve fit to one or two site models ( $p < 0.005$ ). IC<sub>50</sub> values were converted to  $K_i$  values by means of the Cheng and Prusoff equation (Cheng and Pursoff, 1973).

### Figure 6

**Effect of BB-22 administration on DA transmission in the NAc shell, NAc core, and mPFC.**

Results are expressed as mean ± SEM of change in DA extracellular levels expressed as the percentage of basal values. The arrow indicates the start of BB-22 i.v. injection at the dose of 0.003 mg/kg (*magenta triangles*), 0.01 mg/kg (*red triangles*), 0.03 mg/kg (*green squares*), 0.1 mg/kg (*blue diamonds*), or vehicle (*black circles*) in the NAc shell (A), NAc core (B), and mPFC (C). Solid symbol:  $p < 0.05$  with respect to basal values; \* $p < 0.05$  vs veh NAc shell group; ×  $p < 0.01$  vs 0.01 NAc core group; §  $p < 0.01$  vs 0.01 mPFC group; (NAc shell N= 29; NAc core N= 27; mPFC N= 21) (Three-way ANOVA, Tukey's *post hoc*).

### Figure 7

**Blockade of BB-22 effect on increase of DA transmission in the NAc shell by AM 251.**

Results are expressed as mean ± SEM of change in DA extracellular levels expressed as the percentage of basal values. The arrow indicates the start of BB-22 i.v. injection at the dose of 0.01 mg/kg in rats pre-treated with AM 251 (1.0 mg/kg i.p., 30 min before agonist) (*circles*) or vehicle

(triangles). Solid symbol:  $p < 0.05$  with respect to basal values;  $*p < 0.05$  vs veh group. (NAc shell veh N=6; NAc shell AM N=3) (Two-way ANOVA, Tukey's *post hoc*).

### Figure 8

#### Effect of 5F-PB-22, 5F-AKB-48, STS-135 administration on DA transmission in the NAc shell.

Results are expressed as mean  $\pm$  SEM of change in DA extracellular levels expressed as the percentage of basal values. The arrow indicates the start of cannabinoid i.v. injection: (A) 5F-PB-22 0.01 mg/kg (triangles), (B) 5F-AKB-48 0.1 mg/kg (diamonds), and (C) STS-135 0.15 mg/kg (squares), or vehicle (circles) in the NAc shell. Solid symbol:  $p < 0.05$  with respect to basal values;  $*p < 0.05$  vs Veh group (5F-PB-22, N= 6; 5F-AKB-48 N= 7 ; STS-135 N= 5; Veh N=17) (Three-way ANOVA, Tukey's *post hoc*).

**Financial disclosure**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Acknowledgements**

This research has been funded by the European Commission (Drug Prevention and Information Programme 2014-16, contract no. JUST/2013/DPIP/AG/4823, EU-MADNESS project), and by the Fondazione Banco di Sardegna (Prot. U404.2015/AI.338.MGB Prat.2015.0713). The authors would like to thank Dr. Pierluigi Caboni and Dr. Graziella Tocco for the help with the chemical characterization of cannabinoids by GC/MS and NMR analysis.

**References**

Advisory Council on the Misuse of Drugs (ACMD), 2009, Consideration of the Major Cannabinoid Agonists. Home Office, London.

Advisory Council on the Misuse of Drugs (ACMD), 2012, Further Consideration of Synthetic Cannabinoids, Home Office, London

Advisory Council on the Misuse of Drugs (ACMD), 2014, ‘‘Third generation’’ synthetic cannabinoids, Home Office, London

Atwood, B.K., Huffman, J., Straiker, A., Mackie, K., 2010. JWH018, a common constituent of ‘Spice’ herbal blends, is a potent and efficacious cannabinoid CB(1) receptor agonist. *Br. J. Pharmacol.* 160: 585–593.

Atwood, B.K., Lee, D., Straiker, A., Widlanski, T.S., Mackie, K., 2011. CP 47,497-C8 and JWH073, commonly found in ‘Spice’ herbal blends, are potent and efficacious CB(1)cannabinoid receptor agonists. *Eur J Pharmacol.* 659: 139-45.

Auwarter, V., Dargan, P.I., Wood, D.M., 2013. Synthetic Cannabinoid Receptor Agonists. Chapter in: *Novel Psychoactive Substances*. Edited by: Paul I. Dargan and David M. Wood. Elsevier.

Brents, L.K., Reichard, E.E., Zimmerman, S.M., Moran, J.H., Fantegrossi, W.E., Prather, P.L., 2011. Phase I hydroxylated metabolites of the K2 synthetic cannabinoid JWH-018 retain in vitro and in vivo cannabinoid 1 receptor affinity and activity. *PLoS One.* 6, e21917

Brents, L.K., Prather, P.L., 2014. The K2/Spice phenomenon: emergence, identification, legislation and metabolic characterization of synthetic cannabinoids in herbal incense products. *Drug Metab Rev.* Feb; 46(1): 72-85.

Castaneto, M.S., Wohlfarth, A., Desrosiers, N.A., Hartman, R.L., Gorelick, D.A., Huestis, M.A., 2015. Synthetic cannabinoids pharmacokinetics and detection methods in biological matrices. *Drug Metab Rev.* 2015 May;47(2):124-74.

Cheng, Y.C., Prusoff, W.H., 1973, Relationship between the inhibition constant ( $K_i$ ) and the concentration of inhibition which causes 50% inhibition ( $IC_{50}$ ) of an enzyme reaction. *Biochem. Pharmacol.* 22: 3099-3108.

De Luca MA, Solinas M, Bimpisidis Z, Goldberg SR, Di Chiara G. Cannabinoid facilitation of behavioral and biochemical hedonic taste responses. *Neuropharmacology.* 2012 Jul;63(1):161-8. doi: 10.1016/j.neuropharm.2011.10.018. Epub 2011 Nov 2. PubMed PMID: 22063718; PubMed Central PMCID: PMC3705914.

De Luca MA, Valentini V, Bimpisidis Z, Cacciapaglia F, Caboni P, Di Chiara G. Endocannabinoid 2-Arachidonoylglycerol Self-Administration by Sprague-Dawley Rats and Stimulation of in vivo Dopamine Transmission in the Nucleus Accumbens Shell. *Front Psychiatry.* 2014 Oct 17;5:140. doi: 10.3389/fpsy.2014.00140. eCollection 2014. PubMed PMID: 25368584; PubMed Central PMCID: PMC4201088.

De Luca MA, Bimpisidis Z, Melis M, Marti M, Caboni P, Valentini V, Margiani G, Pintori N, Polis I, Marsicano G, Parsons LH, Di Chiara G. Stimulation of in vivo dopamine transmission and intravenous self-administration in rats and mice by JWH-018, a Spice cannabinoid.

Neuropharmacology. 2015 Oct 6;99:705-714. doi:10.1016/j.neuropharm.2015.08.041. [Epub ahead of print] PubMed PMID: 26327678.

Devane, W.A., Dysarz, F.A. 3rd, Johnson, M.R., Melvin, L.S., Howlett, A.C., 1988. Determination and characterization of a cannabinoid receptor in rat brain. *Mol Pharmacol.* 34 :605-613.

Di Chiara G, Bassareo V, Fenu S, De Luca MA, Spina L, Cadoni C, Acquas E, Carboni E, Valentini V, Lecca D. Dopamine and drug addiction: the nucleus accumbens shell connection. *Neuropharmacology.* 2004;47 Suppl 1:227-41. Review. PubMed PMID: 15464140.

EMCDDA, European Monitoring Centre for Drugs and Drug Addiction 2009, Understanding the 'Spice' phenomenon, EMCDDA Thematic Paper, Publications Office of the European Union, Luxembourg.

Huffman, J.W., Zengin, G., Wu, M.J., Lu, J., Hynd, G., Bushell, K., Thompson, A.L., Bushell, S., Tartal, C., Hurst, D.P., Reggio, P.H., Selley, D.E., Cassidy, M.P., Wiley, J.L., Martin, B.R., 2005. Structure–activity relationships for 1-alkyl-3-(1-naphthoyl)indoles at the cannabinoid CB(1) and CB(2) receptors: steric and electronic effects of naphthoyl substituents. New highly selective CB(2) receptor agonists. *Bioorg Med Chem* 13: 89 –112.

Johnston, L.D., O'Malley, P.M., Bachman, J.G. and Schulenberg, J.E., 2013. "Monitoring the Future national survey results on drug use, 1975-2012. Volume I: Secondary school students", Ann Arbor: Institute for Social Research, The University of Michigan, 604 pp.

Lecca D, Cacciapaglia F, Valentini V, Di Chiara G. Monitoring extracellular dopamine in the rat nucleus accumbens shell and core during acquisition and maintenance of intravenous WIN 55,212-2

self-administration. *Psychopharmacology (Berl)*. 2006 Sep;188(1):63-74. Epub 2006 Jul 19. PubMed PMID: 16850116.

Manera, C., Benetti, V., Castelli, M.P., Cavallini, T., Lazzarotti, S., Pibiri, F., Saccomanni, G., Tuccinardi, T., Vannacci, A., Martinelli, A., Ferrarini, P.L., 2006. Design, synthesis, and biological evaluation of new 1,8-naphthyridin-4(1H)-on-3-carboxamide and quinolin-4(1H)-on-3-carboxamide derivatives as CB2 selective agonists. *J Med Chem*. 49: 5947-5957.

Marshall, R., Kearney-Ramos, T., Brents, L.K., Hyatt, W.S., Tai, S., Prather, P.L., Fantegrossi, W.E., 2014. In vivo effects of synthetic cannabinoids JWH-018 and JWH-073 and phytocannabinoid  $\Delta^9$ -THC in mice: inhalation versus intraperitoneal injection. *Pharmacol Biochem Behav*. 124: 40-7.

Papanti, G.D., Orsolini, L., Francesconi, G., Schifano, F., 2014. "Noids" in a nutshell: everything you (don't) want to know about synthetic cannabimimetics. *Advances in Dual Diagnosis* 7: 1-13.

Papanti, D., Schifano, F., Bonavigo, T., Botteon, G., Bertossi, F., Mannix, J., Impagnatiello, M., Pascolo-Fabrizi, E., 2013 'Spiceophrenia': a systematic overview of 'Spice'-related psychopathological issues and a case report. *Human Psychopharmacology: Clinical and Experimental*. 28: 379-89.

Paxinos, G., & Watson, C. (1998). *The rat brain in stereotaxic coordinates*, 4th Ed. Academic Press: San Diego, CA.

Pertwee, R.G., 2005. Pharmacological actions of cannabinoids. *Handb Exp Pharmacol*. 168:1-51. Review. PubMed PMID: 16596770.

Plymouth Herald, 2015. Available from: <http://www.plymouthherald.co.uk>

Pontieri FE, Tanda G, Di Chiara G. Intravenous cocaine, morphine, and amphetamine preferentially increase extracellular dopamine in the "shell" as compared with the "core" of the rat nucleus accumbens. Proc Natl Acad Sci U S A. 1995 Dec 19;92(26):12304-8.

Pontieri FE, Tanda G, Orzi F, Di Chiara G. Effects of nicotine on the nucleus accumbens and similarity to those of addictive drugs. Nature. 1996 Jul 18;382(6588):255-7. PubMed PMID: 8717040.

Santacroce, R., Corazza, O., Martinotti, G., Bersani, F.S., Valeriani, G., Di Giannantonio, M., 2015. Psyclones: a roller coaster of life? Hidden synthetic cannabinoids and stimulants in apparently harmless products. *Hum Psychopharmacol.* 30: 265-271.

Schifano, F., Orsolini, L., Papanti, D., Corkery, J., 2015. Novel psychoactive substances of interest for psychiatry. *World Psychiatry* 14: 15–26.

Simonato, P.L., Corazza, O., Santonastaso, P., Corkery, J., Deluca, P., Davey, Z., Blaszkowski, U., Schifano, F., 2013. Novel psychoactive substances as a novel challenge for health professionals; results from an Italian survey. *Human Psychopharmacology: Clinical and Experimental.* 28: 324-331.

Schlatter, J., 2014. Chapter in *Studies in Natural Products Chemistry*. Edited by Atta-ur- Rahman, Elsevier.

Spaderna, M., Addy, P.H., D'Souza, D.C., 2013. Spicing things up: synthetic cannabinoids. *Psychopharmacology (Berl)*. 228: 525-540.

Tanda G, Pontieri FE, Di Chiara G. Cannabinoid and heroin activation of mesolimbic dopamine transmission by a common mu1 opioid receptor mechanism. *Science*. 1997 Jun 27;276(5321):2048-50. PubMed PMID: 9197269.

Thomas, B.F., Gilliam, A.F., Burch, D.F., Roche, M.J., Seltzman, H.H., 1998. Comparative receptor binding analyses of cannabinoid agonists and antagonists. *J Pharmacol Exp Ther*. 285:285-92.

Uchiyama, N., Kawamura, M., Kikura-Hanajiri, R., Goda, Y., 2012. Identification of two new-type synthetic cannabinoids, N-(1-adamantyl)-1-pentyl-1H-indole-3-carboxamide (APICA) and N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide (APINACA), and detection of five synthetic cannabinoids, AM-1220, AM-2233, AM-1241, CB-13 (CRA-13), and AM-1248, as designer drugs in illegal products. *Forensic Toxicol* 30: 114–125.

Uchiyama, N., Kawamura, M., Kikura-Hanajiri, R., Goda, Y., 2013a. URB-754: a new class of designer drug and 12 synthetic cannabinoids detected in illegal products. *Forensic Sci Int* 227: 21-32.

Uchiyama, N., Matsuda, S., Kawamura, M., Kikura-Hanajiri, R., Goda, Y., 2013b. Two new-type cannabimimetic quinolinyl carboxylates, QUPIC and QUCHIC, two new cannabimimetic carboxamide derivatives, ADBFUBINACA and ADBICA, and five synthetic cannabinoids detected with a thiophene derivative  $\alpha$ -PVT and an opioid receptor agonist AH-7921 identified in illegal

products. *Forensic Toxicol* 31: 223–240.

van Amsterdam, J., Brunt, T., van den Brink, W., 2015. The adverse health effects of synthetic cannabinoids with emphasis on psychosis-like effects. *J Psychopharmacol.* 29(3):254-63.

Vigolo, A., Ossato, A., Trapella, C., Vincenzi, F., Rimondo, C., Seri, C., Varani, K., Serpelloni, G. Marti, M., 2015. Novel halogenated derivatives of JWH-018: behavioral and binding studies in mice. *Neuropharmacology* 95: 68-82.

Wedinos 2014. Philtre issue no. 3. Available from:

[http://www.wedinos.org/resources/downloads/Philtre\\_Issue\\_3.pdf](http://www.wedinos.org/resources/downloads/Philtre_Issue_3.pdf) (accessed on 3rd August 2015)

Wiley, J. L., Marusich, J. A., Martin, B. R., Huffman, J. W., 2012. 1-Pentyl-3-phenylacetylindoles and JWH-018 share in vivo cannabinoid profiles in mice. *Drug and Alcohol Dependence*, 123: 148–53.

Zawilska, J.B., Wojcieszak, J., 2014. Spice/K2 drugs--more than innocent substitutes for marijuana. *Int J Neuropsychopharmacol.* 17(3):509-25.

## Figures

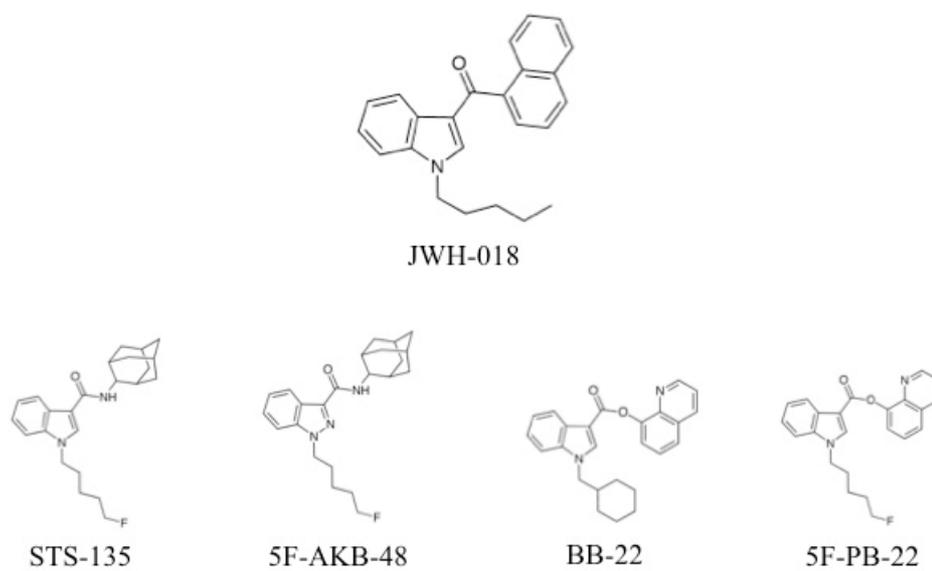


Figure 1

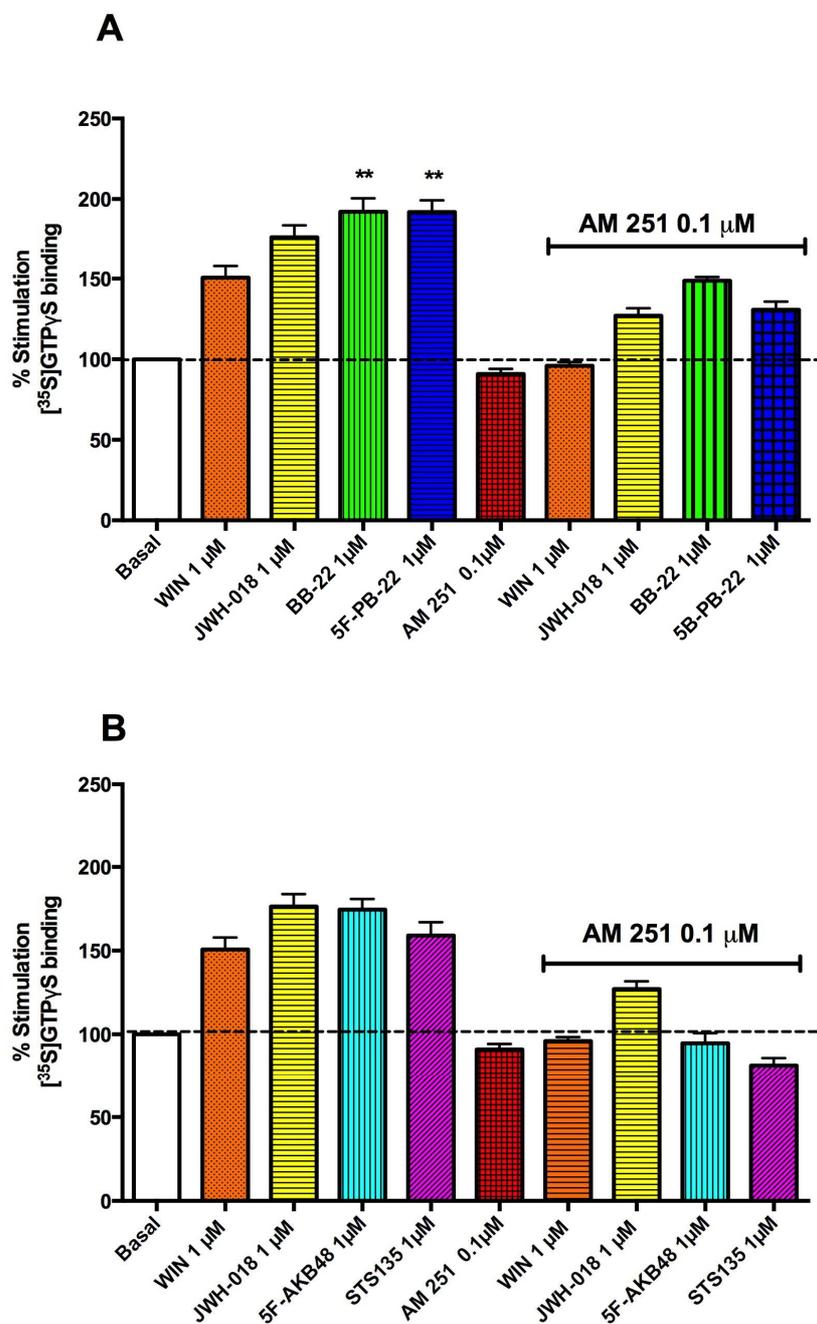


Figure 2

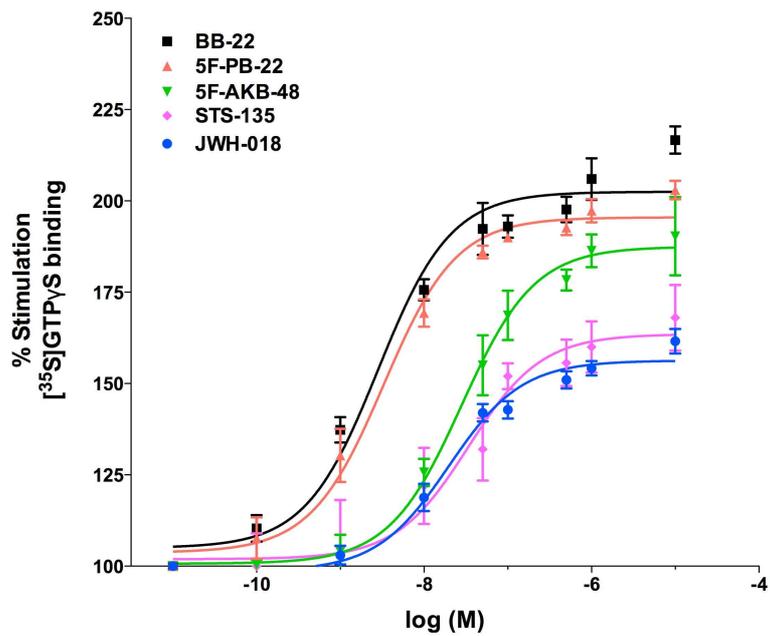


Figure 3

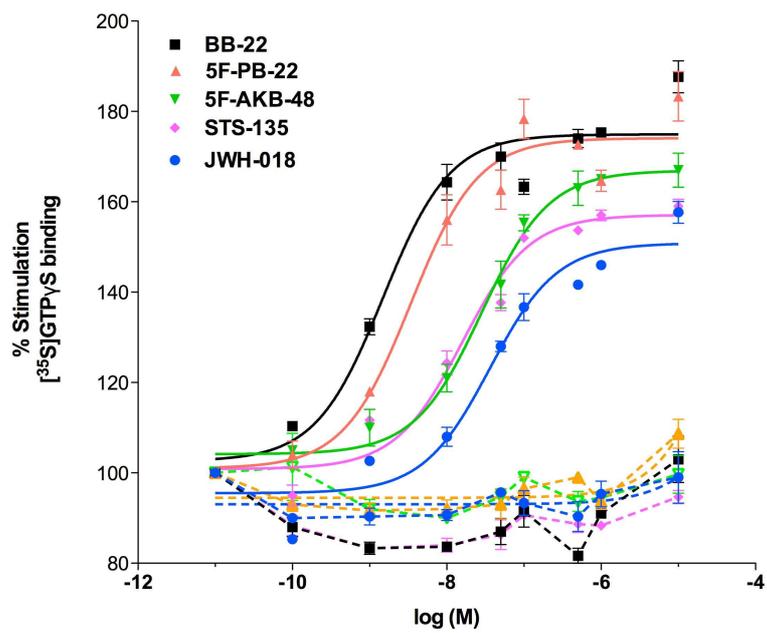


Figure 4

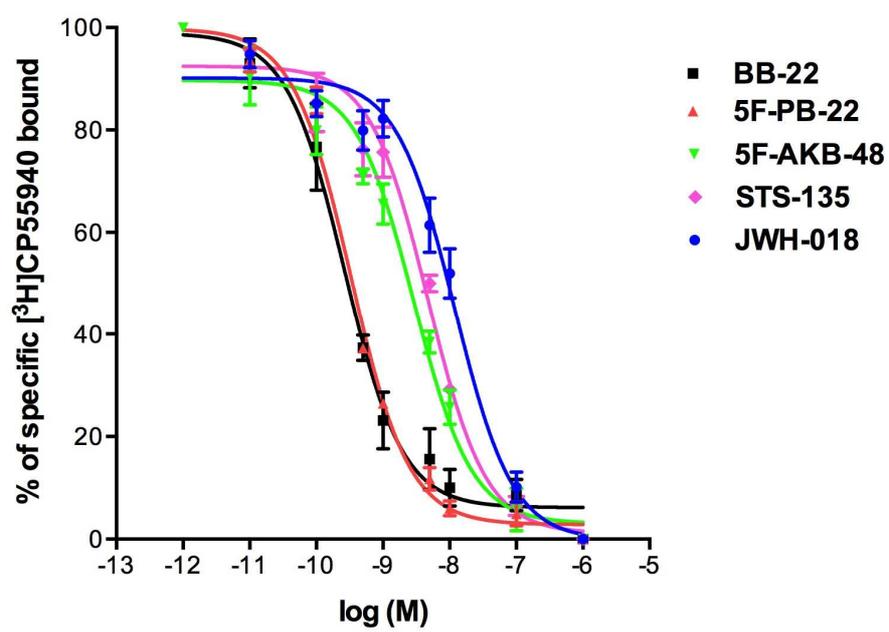


Figure 5

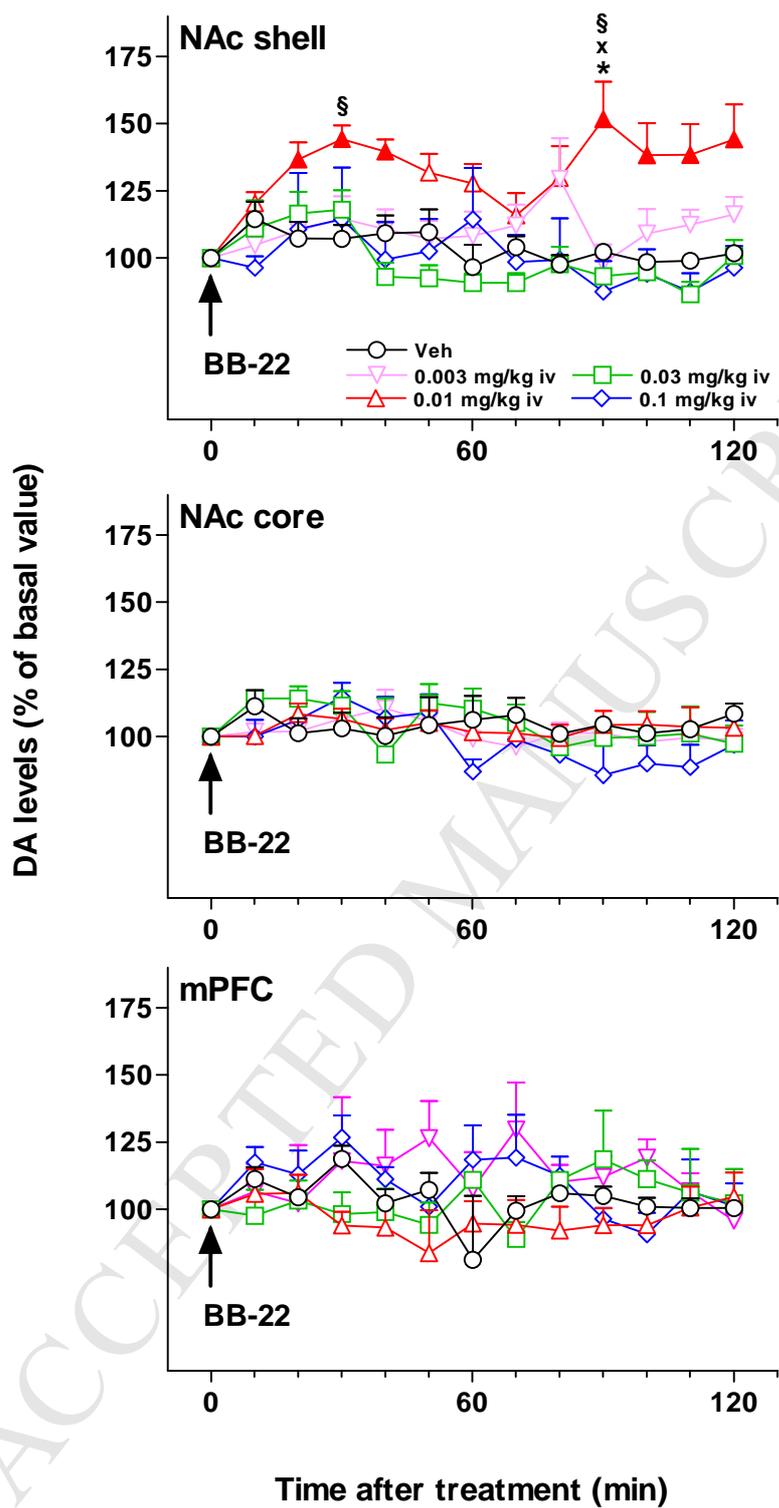


Figure 6

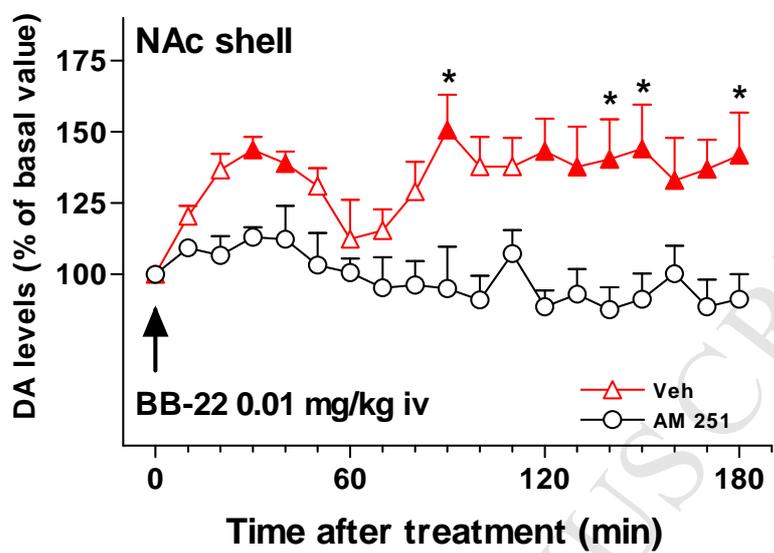


Figure 7

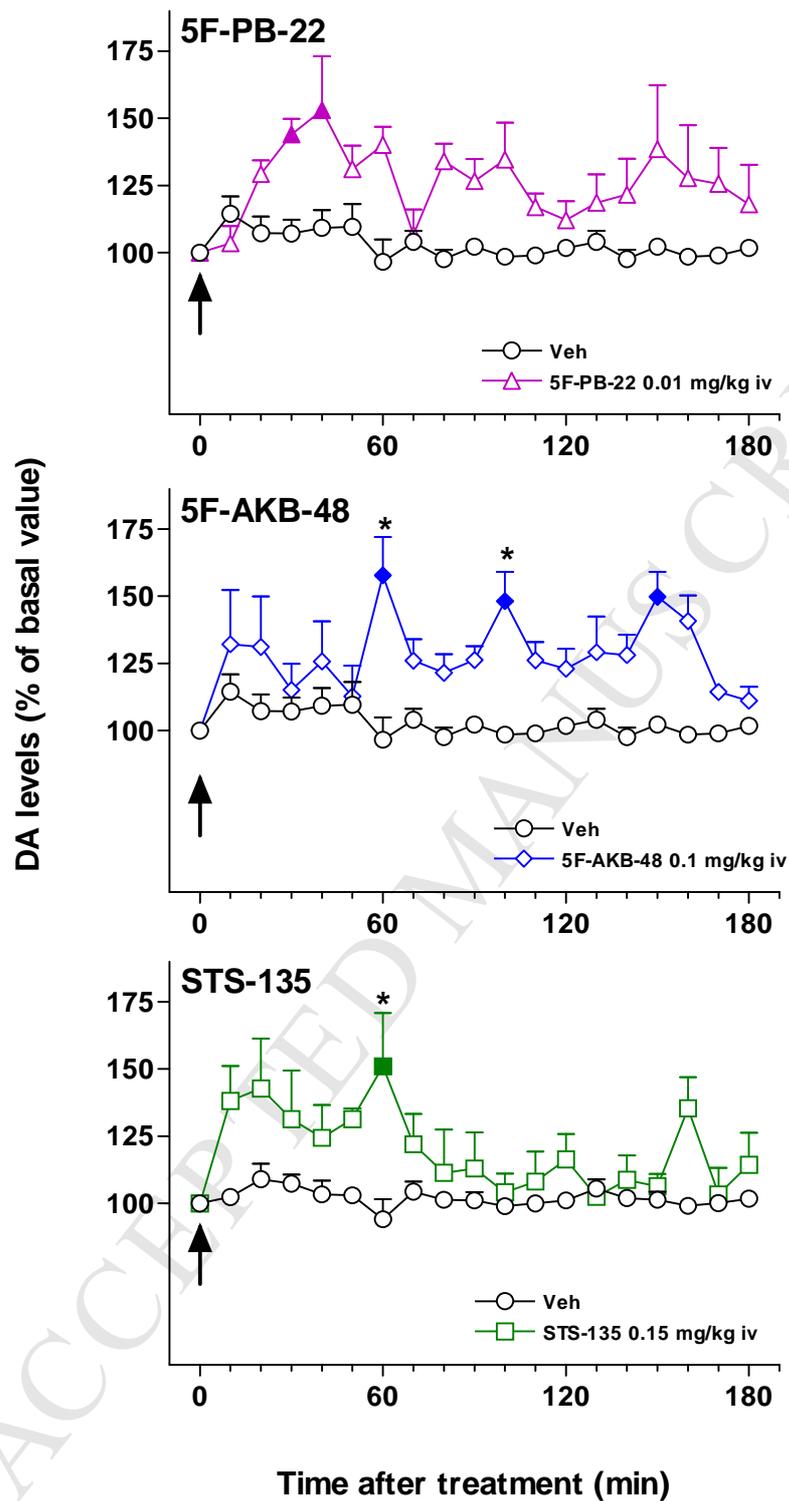


Figure 8

**Table 1. Binding affinity, potency and efficacy for stimulation of [<sup>35</sup>S]GTP $\gamma$ S binding in rat cortical membranes**

Compounds	CB1 Ki (nM)	GTP $\gamma$ S binding	
		EC <sub>50</sub> nM	E <sub>max</sub> % over basal
BB-22	0.11 ± 0.03***	2.9 ± 0.6*	217 ± 4**
5F-PB-22	0.13 ± 0.01***	3.7 ± 0.6*	203 ± 2**
5F-AKB48	0.87 ± 0.14***	31.0 ± 7.5	190 ± 11*
STS-135	1.93 ± 0.18*	32.3 ± 2.9	168 ± 9
JWH-018	3.38 ± 0.63	20.2 ± 1.3	163 ± 3

Data are the means ± SEM of at least four experiments, each performed in triplicate. The calculation of IC<sub>50</sub> was performed by non-linear curve fitting of the concentration-effect curves using Graphpad Prism. IC<sub>50</sub> values were converted to Ki values by means of the Cheng and Prusoff equation (Cheng and Pursoff, 1973). Compound-mediated [<sup>35</sup>S]GTP $\gamma$ S binding data represent percentage of stimulation over basal values (set as 100%). E<sub>max</sub> and EC<sub>50</sub> were determined by non linear regression curve fit (GraphPad Prism). One way ANOVA: Ki: F<sub>(4,14)</sub>=21.24, P<0.0001; EC<sub>50</sub>: F<sub>(4,14)</sub> = 14.78 P<0.0001; E<sub>max</sub>: F<sub>(4,14)</sub> =11.56 P< 0.001 \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 compared to JWH-018 (Dunnett's test).

*Supplementary Material***Characterisation of NPS Internet Products using Nuclear Magnetic Resonance Spectroscopy (NMR)**

Nuclear Magnetic resonance spectra were recorded at 289 K using a Jeol Eclipse-400 spectrometer. The data is reported as chemical shift ( $\delta$ ) in ppm relative to the residual protonated solvent resonance. Relative integral, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), coupling constants are reports in Hz. All deuterated solvents were purchased from Sigma Aldrich (Gillingham, UK). The number of peaks and splitting patterns are consistent with the chemical structures and in line with spectra produced by SWG Drug<sup>1-4</sup>.

**5F-AKB-48**

<sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  (ppm) 1.45-2.20 (21H, m, alkyl), 4.36-4.37 (1H, m, CH<sub>2</sub>), 4.47-4.49 (3H, m alkyl), 6.80 (1H, s, NH), 7.25-7.37 (3H, m, Ar-H), 8.36-8.38 (1H, d,  $J=8$  Hz, Ar-H)

**BB-22**

<sup>1</sup>H-NMR (400 MHz, CD<sub>3</sub>OD)  $\delta$  (ppm) 1.02-1.31 (5H, m, alkyl), 1.62-1.79 (5H, m, alkyl), 1.90-2.03 (1H, m, CH), 4.12-4.13 (2H, d,  $J=4$  Hz, CH<sub>2</sub>), 7.22-7.26 (1H, m, Ar-H), 7.28-7.32 (1H, m Hz, Ar-H), 7.56-7.60 (2H, m, Ar-H), 7.62-7.70 (2H, m, Ar-H), 7.88-7.91 (1H, dd,  $J=7.6, 1.6$  Hz, Ar-H), 8.11-8.14 (1H, dt,  $J=8.1$  Hz, Ar-H), 8.29 (1H, s, Ar-H), 8.40-8.43 (1H, dd,  $J=12$  Hz, Ar-H), 8.82-8.85 (1H, dd,  $J=12$  Hz, Ar-H)

**STS-135**

<sup>1</sup>H-NMR (400 MHz, CD<sub>3</sub>OD)  $\delta$  (ppm) 1.35-1.44 (2H, m, CH<sub>2</sub>), 1.61-1.76 (3H, m, CH<sub>2</sub>), 1.77 (7H, bs, alkyl), 1.85-1.95 (2H, m, CH<sub>2</sub>), 2.10 (3H, bs, alkyl), 2.20 (7H, m, alkyl), 4.21 (2H, t,  $J = 6.7$  Hz, CH<sub>2</sub>), 4.32 (1H, t,  $J = 8$  Hz, CH<sub>2</sub>), 4.43 (1H, t,  $J = 8$  Hz, CH<sub>2</sub>), 6.92-6.96 (1H, br, s, NH), 7.11-7.24 (2H, m, Ar-H), 7.41-7.44 (1H, d,  $J = 8$  Hz, Ar-H), 7.84 (1H, s, CH), 8.00-8.02 (1H, d,  $J=8$  Hz, Ar-H)

**5F-PB-22**

$^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$  (ppm) 1.47-1.57 (2H, m,  $\text{CH}_2$ ), 1.68-1.82 (2H, m,  $\text{CH}_2$ ), 1.96-2.09 (2H, m,  $\text{CH}_2$ ), 4.22-4.24 (2H, t,  $J = 8$  Hz,  $\text{CH}_2$ ), 4.39 (1H, t,  $\text{CH}_2$ ), 4.51 (1H, t,  $\text{CH}_2$ ) 7.27-7.35 (2H, m, Ar-H), 7.41-7.44 (2H, m, Ar-H), 7.54-7.64 (2H, m, Ar-H), 7.75-7.78 (1H, dd,  $J = 7.9, 1.5$  Hz, Ar-H), 8.17 (1H, s, Ar-H), 8.19-8.24 (1H, dd,  $J = 8.5, 1.7$  Hz, Ar-H), 8.29-8.31 (1H, m, Ar-H), 8.90-8.92 (1H, dd,  $J = 4.1, 1.5$  Hz, Ar-H)

References 1-4

<http://www.swgdrug.org/Monographs/5F-AKB48.pdf>

<http://www.swgdrug.org/Monographs/5FPB22.pdf>

<http://www.swgdrug.org/Monographs/5FPB22.pdf>

<http://www.swgdrug.org/Monographs/STS-135.pdf>

***Characterisation of NPS Internet Products using Gas Chromatography Mass Spectrometry with Electron Ionisation (GC-EI-MS).***

HPLC grade methanol was purchased from Fisher Scientific (Loughborough, UK). Samples prepared for chromatography analysis were placed in clear DP ID 2 mL glass vials, fitted with PTFE/silicone septa certified caps. Analysis was done using electron ionisation (EI) with a scan range of 40 - 1000 m/z. Ion trap, manifold and transfer line temperatures were set to 150, 50 and 250 °C, respectively. Gas chromatographic separation was achieved using a Varian FactorFour 5% phenyl-methyl capillary column (30 m x 0.25 mm x 0.25  $\mu\text{m}$ ) using helium as a carrier gas (1 ml  $\text{min}^{-1}$ ) and a split ratio of 10:1. The column was heated to 50 °C for 2 min following sample injection, increased to 300 °C (15 °C  $\text{min}^{-1}$ ), held for 5 min and then cooled back to 50 °C (50 °C  $\text{min}^{-1}$ ), with a total run time of 28.67 min. Solutions of each product were prepared of each product using methanol (0.1 mg  $\text{mL}^{-1}$ ) and filtered (0.2  $\mu\text{m}$  PTFE membrane filters) prior to analysis. Mass

spectra of selected peaks were compared to that of purchased reference standards and the SWG Drug Library (Version 2.1).

The EI fragmentation peaks for the products are reported below as m/z (% abundance) for mass peaks  $\geq 25\%$  abundance.

**5F-AKB-48**

355 (100), 383 (74), 233 (72), 294 (52), 338 (39), 145 (34), 213 (30), 298 (27), and 356 (25%)

**5F-PB-22**

232 (100), 144 (25), and 116 (25%)

**BB-22**

240 (100) and 144 (31%)

**STS-135**

232 (100), 382 (85), 307 (51), 144 (43), and 365 (32%).

***Characterisation of NPS Internet Products using High Performance Liquid Chromatography (HPLC)***

HPLC grade methanol, acetonitrile, water and ortho-phosphoric acid were purchased from Fisher Scientific (Loughborough, UK). Reverse phase HPLC analysis was performed using an integrated Perkin Elmer Flexar system fitted with an in-line degasser, 100-place auto injector and a photodiode array (PDA) detector (recording 210 nm). Data analysis was carried out using Chromera-flexa software version 3.4.0.5712. A core kinetix 5 $\mu$  XB Phenomenex C18 columns (150 x 4.6 mm) was used for the analysis. An Isocratic mobile phase was used of 60% orthophosphoric acid (2.1 pH): 40% AN. The flow rate was 1.5 mL min<sup>-1</sup> with an injection volume of 5  $\mu$ L. Stock solutions of each NPS were prepared by adding 5.0 mg of each component weighed accurately into 10 mL volumetric flasks and made up to volume with methanol. Each NPS sample was prepared and analysed in triplicate. A calibration curve was constructed using the following standards: 1, 0.66, 0.44, 0.29, 0.19, and 0.13 mg ml<sup>-1</sup>.

HPLC was used to run the reference standards 5F-AKB-48 (RT = 16.3 min), 5F-PB-22 (RT = 3.4 min), BB-22 (RT = 8.9 min), STS-135 (RT = 9.2 min) and evaluate the purity of the Internet products. Correlation coefficients for all calibration curves were  $\geq 0.999$ . The purity of the 5F-AKB-48, 5F-PB-22, BB-22, and STS-135 Internet products were determined to be  $93 \pm 1\%$  (RT = 16.2 min),  $95.2 \pm 0.8\%$  (RT = 3.4 min),  $90.6 \pm 0.6\%$  (RT = 8.9 min), and  $91 \pm 2\%$  (RT = 9.2 min), respectively.

**HIGHLIGHTS**

- STS-135, 5F-AKB-48, BB-22, 5F-PB-22 are 3<sup>rd</sup> generation Spice/K2 synthetic cannabinoids
- They are CB1 receptor ligands with higher affinity than JWH-018
- They are CB1 agonists with higher potency and intrinsic activity than JWH-018
- They increase NAc shell dopamine at doses consistent with their *in vitro* potency