Materials and Methods

Chemicals and Drugs

The non-selective CB1R/CB2R agonists JWH-018 [(1-pentyl-1H-indol-3-yl)-1-naphthalenyl-methanone), Δ^9-THC (Δ^9-tetrahydrocannabinol) and 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 mesylate] were purchased from Cayman Chemical (Ann Arbor, MI), Cerilliant (Round Rock, TX) and Tocris Bioscience (Ellisville, MO), respectively. JWH-018 N-(5-hydroxypentyl) β-D-glucuronide, a major metabolite of JWH-018, was synthesized by Cayman Chemical Company where ^{1}HNMR analysis indicates chemical shifts and coupling constants consistent with the chemical structure (Figure 1 and Figure 2). (+)-11-Nor-Δ^9-THC-9-carboxylic acid glucuronide is a major metabolites of THC and was synthesized by Cerilliant. O-2050 [(6a*R*,10a*R*)-3-(1-Methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6*H*-dibenzo[*b*,*d*]pyran)] is a neutral antagonist at CB1Rs and was purchased from Tocris Bioscience. [^{3}H]CP-55,940 is a non-selective CB1/CB2R agonist used for competition receptor binding assays and was purchased from Perkin Elmer Life and Analytical Sciences (Wellesey, MA). [^{35}S]GTPγS was used to quantify G-protein activation and was purchased from American Radiolabeled Chemicals (St. Louis, MO). Non-radiolabeled GTPγS was purchased from EMD Chemical (Gibbstown, NJ).

Mice

The University of Arkansas for Medical Sciences IACUC committee approved the animal use protocol utilized in this study. B6SJL mice were maintained on a 12 hour light/dark cycle with free access to food and water. Mice were sacrificed under isoflurane anesthesia and

brains were harvested, snap frozen in liquid nitrogen and stored at -80^{o}C until membrane homogenates were prepared.

Brain Membrane Preparation

Whole mouse brains were homogenized using a Dounce glass homogenizer in an ice-cold buffer comprised of 50 mM HEPES pH 7.4, 3 mM $MgCl_2$, and 1 mM EDTA. Homogenates were centrifuged at 40,000 x *g* for 10 minutes at 4°C and the supernant discarded. Pellets were homogenized and centrifuged similarly twice more and finally suspended in 50 mM HEPES, pH 7.4, divided into aliquots and stored at -80°C.

Competition Receptor Binding Assays

Receptor binding assays utilized 50 µg of mouse brain membranes as the source of CB1Rs and the non-selective CB1R/CB2R radioligand [^{3}H]CP-55,940, as previously described[1]. Briefly, each binding reaction was conducted in triplicate and was performed in a total volume of 1 ml that contained a final concentration of 0.2 nM [^{3}H]CP-55,940, 50 mM Tris (pH 7.4), 0.05% bovine serum albumin, 5 mM $MgCl_2$, 1.0% DMSO, 0.1% EtOH and increasing concentrations (0.1 nM – 10 µM) of either JWH-018, THC, 018-gluc, or THC-gluc. Non-specific binding was defined as the amount of [^{3}H]CP-55,940 binding remaining in the presence of a 10 µM concentration of the high affinity, non-radioactive CB1R/CB2R agonist WIN-55,212-2. After the addition of all reagents, all samples were mixed by vortexing and incubated at room temperature for 1.5 h to allow the reaction to reach equilibrium binding. Binding reactions were terminated by rapid vacuum filtration through glass fiber filters with three washes of ice-cold 50 mM Tris pH 7.4 containing 0.05% bovine serum albumin. The amount of radioactivity for each sample

was quantified by scintillation counting the following morning by adding 4 ml of Scintiverse scintillation fluid (Fisher Scientific, Waltham, MA) to each filter paper.

GTPγS Binding Assays

G-protein activity was quantified by employing 25 μg of mouse brain membranes and [^{35}S]GTPγS in a total reaction volume of 1 ml, as previously described[2]. Specifically, the ability of all cannabinoid compounds to modulate G-protein activity was examined in triplicate samples in a buffer containing a final concentration of 0.1 nM [^{35}S]GTPγS, 20 mM HEPES (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, 10 μM GDP, 0.05% bovine serum albumin, 20 units/L adenosine diaminase, 0.1% EtOH and 1.0% DMSO. Nonspecific binding was defined by the amount of [^{35}S]GTPγS binding remaining in the presence of a 10 μM concentration of non-radiolabeled GTPγS. Reaction mixtures were thoroughly mixed by vortexing and incubated at 30°C for 30 minutes. Rapid vacuum filtration through glass fiber filters terminated reactions and all samples were subsequently washed three times with ice-cold 50 mM HEPES (pH 7.4) containing 0.05% bovine serum albumin. The amount of radioactivity for each sample was quantified by scintillation counting the following morning after adding 4 ml of Scintiverse scintillation fluid to the filter papers.

Statistical Analysis

GraphPad Prism® v4.0b (GraphPad Software, Inc., San Diego, CA) was used for all curve-fitting and statistical analyses. Data from three of more experimental groups were analyzed by one-way ANOVA followed by a Newman-Keuls Multiple Comparison *post-hoc*

comparison. The non-paired Student's *t*-test was used to compare data from two experimental groups. Statistical significant differences were defined as $P < 0.05$.

Supplemental References

(1) Prather, P. L., Song, L., Piros, E. T., Law, P. Y. and Hales, T. G. (2000) delta-Opioid receptors are more efficiently coupled to adenylyl cyclase than to L-type Ca(2+) channels in transfected rat pituitary cells. *J Pharmacol Exp Ther 295*, 552-562.

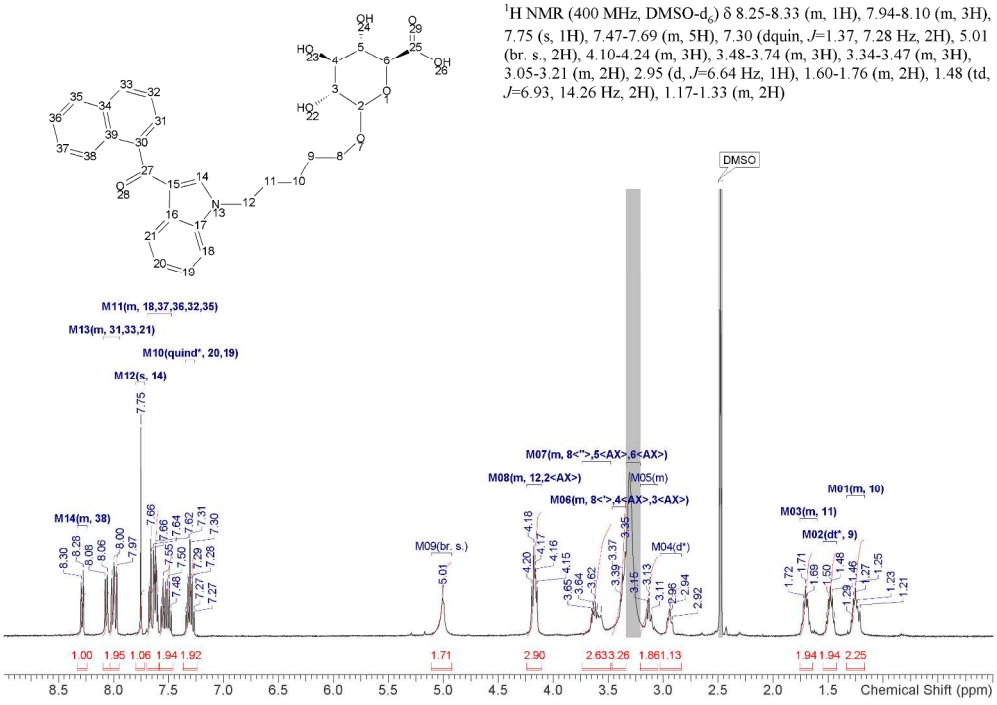
(2) Prather, P. L., Martin, N. A., Breivogel, C. S. and Childers, S. R. (2000) Activation of cannabinoid receptors in rat brain by WIN 55212-2 produces coupling to multiple G protein alpha-subunits with different potencies. *Mol Pharmacol 57*, 1000-1010.

Figure Legends

Figure 1: ^{1}HNMR confirmation data for JWH-018 N-(5-hydroxypentyl) β-D-glucuronide.

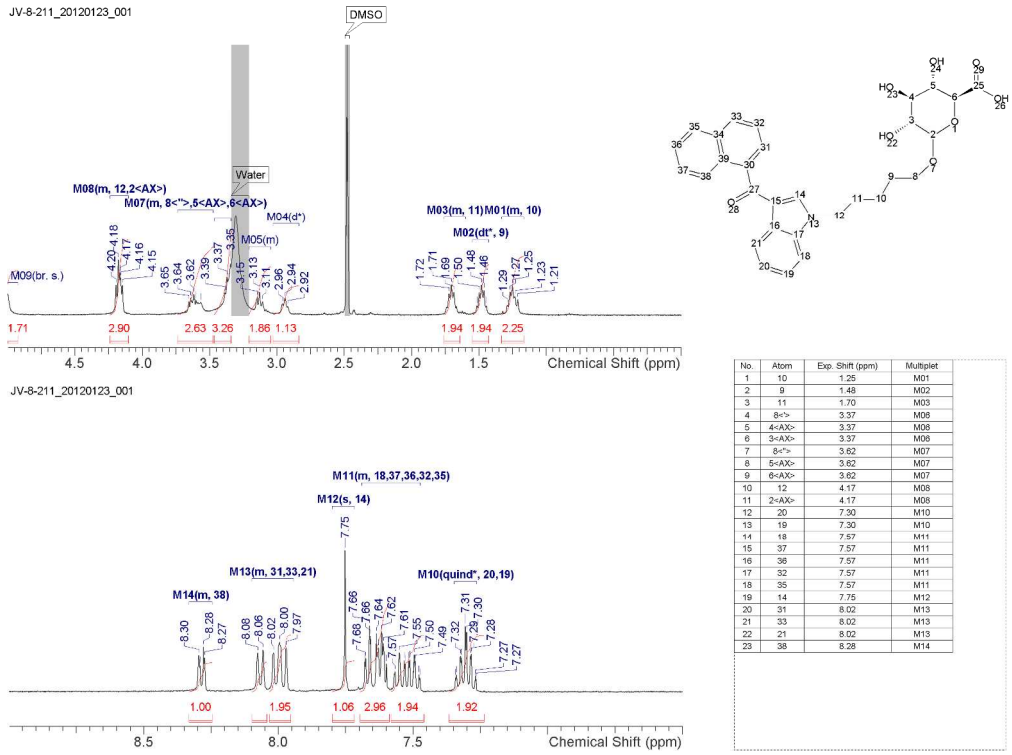
Figure 2: ^{1}HNMR confirmation data for JWH-018 N-(5-hydroxypentyl) β-D-glucuronide.

JWH-018-N-(5-hydroxypentyl)-β-D-glucuronide



^{1}H NMR (400 MHz, DMSO-d_6) δ 8.25-8.33 (m, 1H), 7.94-8.10 (m, 3H), 7.75 (s, 1H), 7.47-7.69 (m, 5H), 7.30 (dquin, *J*=1.37, 7.28 Hz, 2H), 5.01 (br. s., 2H), 4.10-4.24 (m, 3H), 3.48-3.74 (m, 3H), 3.34-3.47 (m, 3H), 3.05-3.21 (m, 2H), 2.95 (d, *J*=6.64 Hz, 1H), 1.60-1.76 (m, 2H), 1.48 (td, *J*=6.93, 14.26 Hz, 2H), 1.17-1.33 (m, 2H)

794x592mm (96 x 96 DPI)



No.	Atom	Exp. Shift (ppm)	Multiplet
1	10	1.25	M01
2	9	1.48	M02
3	11	1.70	M03
4	8<">	3.37	M08
5	4<AX>	3.37	M08
6	3<AX>	3.37	M08
7	8<">	3.62	M07
8	5<AX>	3.62	M07
9	6<AX>	3.62	M07
10	12	4.17	M08
11	2<AX>	4.17	M08
12	20	7.30	M10
13	19	7.30	M10
14	18	7.57	M11
15	37	7.57	M11
16	36	7.57	M11
17	32	7.57	M11
18	35	7.57	M11
19	14	7.75	M12
20	31	8.02	M13
21	33	8.02	M13
22	21	8.02	M13
23	38	8.28	M14

252x188mm (300 x 300 DPI)