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SYMPOSIUM ON THE CANNABINOIDS

ASILOMAR CONFERENCE CENTER
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Program and Abstracts

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2002 SYMPOSIUM OVERVIEW			
TIME	**THURSDAY JULY 11TH**	**FRIDAY JULY 12TH**	**SATURDAY JULY 13TH**
All ICRS Meetings are in Chapel unless otherwise noted			
7:00	**Breakfast**	**Breakfast**	**Breakfast**
8:00	**SAR and Ligand Interactions** Chairs: MacAllister/Seltzman Page 1	**Physiological Roles of Cannabinoids** Chairs: Howlett/Maccarone Page 26	**Food for Thought** Chair: Guy Page 46
8:45			**Therapeutic Potential** Chair: Pertwee
9:30	**Coffee**	**Coffee**	
9:45			**Coffee**
10:00	**Endocannabinoids** Chairs: Cravatt/DiMarzo Page 7		
10:15			**Human Studies—Therapeutic Potential** Chair: Fernández-Ruiz Page 53
10:30		**Learning, Memory** Chair: Litchman/Lupica Page 34	
11:00		**Appetite and Feeding** Chair: Kunos Page 38	
11:30			**Human Studies—Adverse Effects** Chair: Parolaro Page 58
12:30		**Lunch**	**Lunch**
12:45	**Lunch**		
1:15			**NIDA Info Luncheon Workshop** Taking Your Product to Market

			Speakers: Kirk Maxey/Brian Thomas
2:00	**Joint Session ICRS/INRC THC – Opioid Receptor Function Regulation** Chairs: Chavkin/Mackie **Merrill Hall** Page 18	**Immune Regulation** Chair: Klein Page 41	**Joint Session ICRS/INRC New Possibilities for Opioids and Cannabinoids** Chair: Keiffer **Merrill Hall** Page 62
3:25		**Coffee**	
4:15		**Business Meeting**	
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4:30		**Poster Session** Learning and Memory Electrophysiology, Immune Functions, Pain and Inflammation, Appetite and Feeding, Motor Functions, Hormones Pages 103 - 133	
4:30	**Poster Session Coffee** Structure-Activity Relationships and Endocannabinoids Pages 69 - 109		**Poster Session Coffee** Neuroprotection, Therapeutic Potential, Adverse Effects, Tolerance and Dependence Pages 134 - 161
6:00	**Dinner**	**Dinner on the Meadow**	**Dinner**
7:30	**Kang Tsou Memorial Lecture – Richard Tsien** Understanding How CNS Synapses Remodel Themselves **Merrill Hall**		

12th Symposium On The Cannabinoids
2002 Program

Wednesday July 10th – Registration and Reception

Thursday July 11th

7:00	**Breakfast** 		
SAR and Ligand Interactions ➢*Chairs: McAllister/Seltzman*			
8:00	Razdan, R.K., Altundas, R., Mahadevan, A., Jefferson,R., Winckler, R., and Martin, B.R.	Anandamide: Further Examples of Analogs which are Potent in the Tetrad Tests and Bind Poorly to the CB_1 Receptor	1
8:15	Martin, B., Stevenson, L.A., Pertwee, R.G., Breivogel, C.S., Williams, W., Mahadevan, A. and Razdan, R.	Agonists and Silent Antagonists in a Series of Cannabinoid Sulfonamides	2
8:30	Huffman, J.W., Miller, J.R.A., Liddle, J., Yu, S, Thomas, B.F., Wiley, J.L., Martin, B.R.	Structure-Activity Relationships for 1',1'-Dimethylalkyl-Δ^8-Tetrahydrocannabinols	3
8:45	Picone, R.P., Ayotte, L.A., Hurst, D., Reggio, P.R., Khanolkar, A.D., Fournier, D.J., Makriyannis, A.	Identification of an Amino Acid in Helix Six involved with CB_1-Ligand Interactions using a Combined Chemistry/Site-Directed Mutagenesis Approach	4
9:00	Thomas, B.F., Francisco, M.E., Seltzman, H.H., Gilliam, A.F., Pertwee, R.G., and Stevenson, L.A.	Analogs of the CB_1 Antagonists SR141716 that Selectively Displace Cannabinoid Agonists	5
9:15	Reggio, P., Hurst, D., Buehner, K., Norris, J.B., McAllister, S.D., and Abood, M.E.	The TMH 3-4-5-6 Aromatic Rich Region of CB_1: Importance of Aromatic Stacking Interactions for the Binding of SR141716A and WIN55,212-2 and C-H•••π Interactions for the Binding of Anandamide	6
9:30	**Coffee** 		
Endocannabinoids ➢*Chairs: Cravatt/DiMarzo*			
10:00	Romero, J., Tolón, Treviño, M.A., Benito, C., Núñez, and Rábano, A.	Cellular Localization of CB_1 and Fatty Acid Amide Hydrolase mRNAs in Selected Areas of the Human Central Nervous System	7
10:15	Sugiura, T., Oka, S., and Waku, K.	Physiological Significance of 2-Arachidonoylglycerol as a Neuromodulator	8
10:30	Glaser, S.T., Studholme, K.M., Fatade, F., Yazulla, S., Abumrad, N., and Deutsch, D.G.	Is there an Anandamide Transporter?	9
10:45	Hillard, C.J., Patel, S., Radamacher, D.J., Carrier, E.J., Perry, L.J., Wohlfiel, E., Nithipatikom, K., and Campbell, W.B.	Endocannabinoids: Novel Mediators of General Anesthetic Action	10
11:00	Sauer,J-M., Baker Leese, A., Felder, C.C., and Porter, A.C.	Further Characterization of the Novel Endocannibinoid Virodhamine	11

11:15	Murillo-Rodriguez, E., Blanco-Centurion, C., Gerashchenko, D., Sanchez, C., Piomelli, D., and Shiromani, P.J.	Anandamide Enhances Extracellular Levels of Adenosine and Induces Sleep in Young Rats: And *In vivo* Microdialysis Study	12
11:30	Maccarrone, M., Cecconi, S., Rossi, G., Battista, N., Bari, M., and Finazzi-Agrò, A.	Ageing and FSh Regulate the Degradation and Pro-Apoptotic Activity of Anandamide in Mouse Sertoli Cells	13
11:45	Smart, D., Jonsson, K-O., Vandevoorde, S., Lambert, D.M., and Fowler, C.J.	*N*-Acylethanolamines Potentiate the Calcium Influx Response to Anandamide in hVR1-HEK293 Cells Expressing Vanilloid Receptors	14
12:00	Burstein, S.H., Huang, S.M., Karim, P., Pearson, W., Petros, T.J., Rossetti, R.G., Walker, J.M., and Zurier, R.B.	Actions of N-Arachidonylglycine on Inflammation and Regulation of Anandamide Degradation	15
12:15	Huang, S.M., Bisogno, T., Petros, T.J., Krey, J.F., Chu, C.J., Miller, J.D., Di Marzo, V., and Walker, J.M.	Isolation, Identification and Pro-Nociceptive Actions of *N*-Arachidonoyl Dopamine (NADA), an Endogenous Molecule Active at VR1/CB_1 Receptors	16
12:30	Bisogno, T., Huang, S.M., Trevisani, M., Al-Hayani, A., De Petrocellis, L., Fezza, F., Tognetto, M., Davies, S.N., Geppetti, P., Walker, J.M., and Di Marzo, V.	An Endogenous Capsaicin-Like Compound that Activates Both Vanilloid and Cannainoid Receptors	17
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Joint Session ICRS/INRC

THC – Opioid Receptor Function Regulation

➤*Chairs: Chavkin/Mackie*

2:00 Introduction **Merrill Hall**

2:05	Von Zastrow, M., Kim, K., Haberstock-Debic, H., Wein, M., Neve, R., Barrot, M., Nestler, E., and Svingos, A.	Membrane Trafficking of Opioid Receptors in Neural Cells	18
2:25	Childers, S.	Chronic Drug Effects of Opioid and Cannabinoid Receptor Activation of G-Proteins in Brain	19
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3:50	Szabo, B., Siemes, S., and Wallmichrath, I.	Depression of GABAergic Neurotransmission by Cannanbinoinds in the Ventral Tegmental Area	24
4:05	Rubino, T., Viganò, D., Zippel, R., and Parolaro, D.	Map Kinases Pathway Plays a Role in Cannabinoid Tolerance	25
4:30-6:00	**Poster Session** **Coffee**  *Structure-Activity Relationships And Endocannnabinoids*		69 to 102
6:00	**Dinner** 		

7:30	**Kang Tsou Memorial Lecture – Richard Tsien** *Understanding How CNS Synapses Remodel Themselves*

Friday July 12th

7:00	**Breakfast** 		
Physiological Roles of Cannabinoids ➤*Chairs: Howlett/Maccarrone*			
8:00	Wallace, M.J., Blair, R.E., Razvi, B., Martin, B.R., and DeLorenzo, R.L.	CB_1 Receptor-Dependent Modulation of Seizure Frequency and Duration in Epileptic Rats: Implications for the Endocannabinoid System in the Treatment of Epilepsy	26
8:15	Fong, T.M., Rycroft, W., Van der Ploeg, L.H.T., and Hutson, P.H.	Effects of the CB_1 Receptor Antagonist AM251 in Induced-Seizure in Rodents	27
8:30	Laine, K., Järvinen, K., Mechoulam, R., Breuer, A., and Järvinen, T.	Evaluation of the Enzymatic Stability and the Intraocular Pressure Erfects of 2-AG and Noladin Ether	28
8:45	Rademacher, D.J., Patel, S., Dean, C., Hopp, F.A., Hillard, C.J., and Seagard, J.L.	Cannabinoid Microinjection into the Nucleus Tractus Solitarius Affects Baroreceptor-Mediated Control of Dynamic Arterial Blood Pressure Changes	29
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9:15	Van Sickle, M., Oland, L., Mackie, K., Davison, J., and Sharkey, K.	Central Site of Action for Cannabinoids as Anti-Emetics	31
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10:15	Gardner, B., Zhu, L.X., Sharma, S., Tashkin, D.P., and Dubinett, S.M.	Methanandamide Treatment Increases Murine Lung Cancer Growth in Immunocompetent Mice by a Cannabinoid Receptor Independent Pathway	33
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3:15	**Coffee** 		
4:15	**Business Meeting**		
4:30-6:00	**Poster Session** *Learning and Memory* *Electrophysiology, Immune Functions, Pain and Inflammation, Appetite and Feeding, Motor Functions, Hormones*		103 to 133
6:00	**Dinner with INRC on the Meadow** 		

Saturday July 13th

7:00	**Breakfast** 		
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12:30	**Lunch** 		
1:15	**NIDA Info Luncheon Workshop** ➤*Chair: Allyn Howlett* ***Taking Your Product to Market*** *Speakers: Kirk Maxey, President, Cayman Chemical Company* Brian Thomas, Senior Research Pharmacologist, *Research Triangle Institute*		
Joint Session ICRS/INRC New Possibilities for Opioids and Cannabinoids ➤*Chair: Keiffer* **2:00 Introduction** **Merrill Hall**			
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4:30-6:00	**Poster Session** **Coffee**  *Neuroprotection, Therapeutic Potential, Adverse Effects, Tolerance and Dependence*		134 to 161
6:00	**Dinner** 		

4:30-6:00	**Thursday, July 11** **Poster Session** *Structure-Activity Relationships And Endocannnabinoids*		69 to 102
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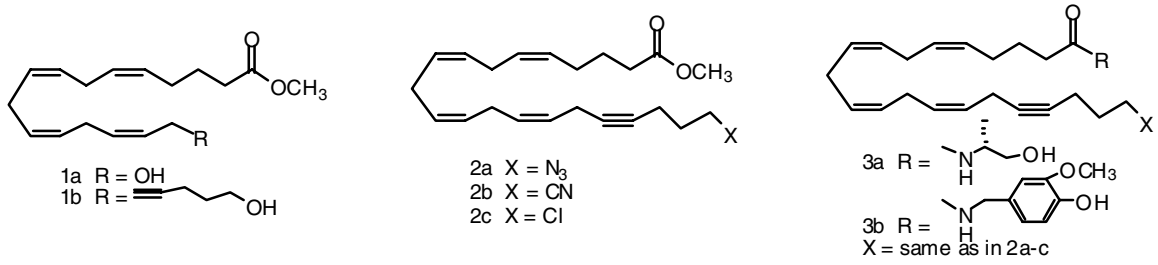
ANANDAMIDE: FURTHER EXAMPLES OF ANALOGS WHICH ARE POTENT IN THE TETRAD TESTS AND BIND POORLY TO THE CB_1 RECEPTOR

Raj K. Razdan,[1] Ramazan Altundas,[1] Anu Mahadevan,[1]
Renee Jefferson,[2] Ramona Winckler[2], and Billy R. Martin[2]
[1]Organix Inc. 240 Salem Street, Woburn, MA 01801, USA
[2]Department of Pharmacology and Toxicology, VCU, Richmond, VA 23298, USA

We have synthesized and tested several other novel analogs of the endogenous ligand anandamide (AEA) in continuation of our studies on the role of its interaction with the cannabinoid receptor CB_1 and the vanilloid receptor VR1 (Di Marzo et al.,Biochem. Biophys. Res. Commun. **2001**, *281*, 444-451; Di Marzo et al., J. Pharmacol. Exp. Ther. **2002**, *300*, 984-941). The present compounds **3** were designed to study the Structure Activity Relationships (SAR) in the series where the end n-pertly chain of AEA has an acetylene group in the chain and carries various substituents at the terminal end of the chain. Similar compounds in the THC series had shown very interesting pharmacological properties and varied in activity as partial agonists to antagonists (Martin et al.,J. Pharmacol. Exp. Ther. **1999**, *290*, 1065-1079).

For the synthesis of these analogs we developed the synthon **1a** which was synthesized from one of the intermediates described by us previously (Dasse, et al., Tetrahedron, **2000**, *56*, 9195-9202). The synthon **1a**, via its bromo derivative (PPh_3, CBr_4) was treated with 4-pentyn-1-ol (CuI, NaI, K_2CO_3, DMF) to give **1b**, which as its mesylate, was treated with NaN_3 in DMF to give **2a**. The methyl ester in **2a** was hydrolyzed (LiOH, MeOH) and then converted to the target analogs **3a** ($X=N_3$) or **3b** ($X=N_3$) using the methodology used by us before (Dasse et al., see above). Similarly, treatment of the bromo derivative of **1a** on treatment with 5-cyano-1-pentyne formed **2b** and with 5-chloro-1-pentyne gave **2c**. Both **2b** and **2c** were similarly transformed to the target compounds **3a** and **3b** as in the case of **2a**. All the compounds in the **3b** series showed very potent in vivo activity ($ED_{50,s}$ in the range of 0.1 to 0.64 mg/kg in various tests) in spite of very poor binding affinities for the CB_1 receptor (739 to 1054 nM range). The details of the total synthesis and the SAR of these analogs will be presented.



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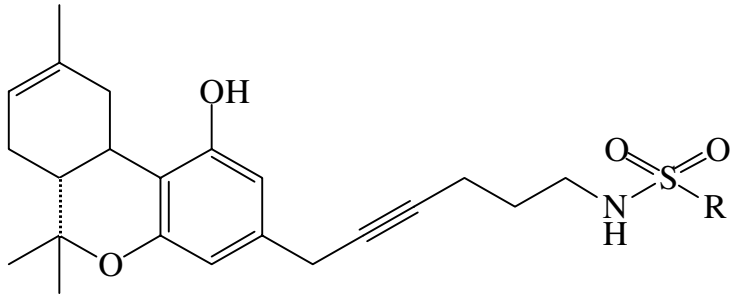
AGONISTS AND SILENT ANTAGONISTS IN A SERIES OF CANNABINOID SULFONAMIDES

Billy R. Martin[1], Lesley A. Stevenson[2], Roger G. Pertwee[2],
Christopher S. Breivogel [3], W. Williams[4], Anu Mahadevan[4], and Raj K. Razdan[4]
[1]Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA, USA; [2]Department of Biological Sciences, Institute of Medical Sciences, University of Aberdeen, Aberdeen Scotland; [3]Department Pharmaceutical Sciences, Campbell University, Buies Creek, NC, USA, [4]Organix Inc., Woburn, MA, USA

The side chain in tetrahydrocannabinol (THC) plays a crucial role in both receptor recognition and activation. Previously, we demonstrated that incorporation of acetylene into the side chain produce greater attenuation in receptor activation than affinity. Moreover, addition of various substituents in the terminal carbon of the acetylenic side chain resulted in high affinity compounds with low to high efficacy. We have extended these studies by preparing sulfonamides as depicted below in which R is either methyl, ethyl, or butyl. The ethyl analog exhibited CB_1 receptor affinity (Ki = 30 nM) and pharmacological potency (ED_{50}'s of 1.7, 0.9, and 0.8 mg/kg for suppression of spontaneous activity, analgesia in the tail-flick assay, and reduction in body temperature, respectively, by i.v. injection) similar to that of THC. The butyl exhibited CB_1 receptor affinity (Ki = 70 nM) and pharmacological potency (ED_{50}'s of 7.5, 14.0 and 11.6 mg/kg in the above assays) that was considerably less than that of THC. Therefore, the ethyl and butyl analogs appear to be typical cannabinoid agonists with the ethyl derivative having higher affinity and pharmacological potency. The methyl derivative exhibited the highest CB_1 receptor affinity with a Ki of 2.5 nM. Interestingly, it produced depression of spontaneous activity (ED_{50} = 4.3 mg/kg) but the doses were higher than that expected of a high affinity CB_1 receptor ligand. This analog failed to produce either antinociception or hypothermia. Therefore, this analog was examined the mouse vas deferens where it was also found to be devoid of agonist effects. However, it was found to be an antagonist. Moreover, it failed to elicit any inverse agonism even at high concentrations. Therefore, this methyl derivative is a 'silent' antagonist in the mouse vas deferens assay. This series of sulfonamides should be useful for delineating the structural features of agonists, antagonists and 'silent antagonists'.



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STRUCTURE–ACTIVITY RELATIONSHIPS FOR 1',1'-DIMETHYLALKYL–Δ^8–TETRAHYDROCANNABINOLS

John W. Huffman[1], John R. A. Miller[1], John Liddle[1], Shu Yu,
Brian F. Thomas[2], Jenny L.Wiley[3], and Billy R. Martin[3]
[1]Department of Chemistry, Clemson University, Clemson, SC 29634, USA
[2]Research Triangle Institute, PO Box 12194, Research Triangle Park, NC 27709, USA
[3]Department of Pharmacology and Toxicology, Medical College of Virginia Campus,
Virginia Commonwealth University, Richmond, VA 23298, USA

A body of empirical structure-activity relationships (SAR) has been developed for cannabinoids structurally related to Δ^9-tetrahydrocannabinol (Δ^9-THC). These SAR point to the importance of a phenolic hydroxyl group at C-1, an alkyl side chain at C-3, natural absolute stereochemistry and substituent effects at C-9 in determining cannabinoid receptor affinity and potency. The nature of the alkyl chain has a profound effect upon the CB_1 receptor affinity and *in vivo* pharmacology of analogs of both Δ^8-THC and Δ^9-THC. There is an incremental increase in both receptor affinity and *in vivo* potency as the length of the side chain is increased form four to eight carbons (Martin *et al. J. Pharmacol. Exp. Ther.* **1999**, *290*, 1065.). It is well known that a 1',1'-dimethylheptyl side chain provides greatly enhanced potency, but the effect of alkyl chain length in the 1',1'-dimethylalkyl-Δ^8- or Δ^9-THC series has not been investigated.

We have prepared the series of 1',1'-dimethylalkyl-Δ^8-THC homologues with side chains of two to twelve carbon atoms, determined their affinity for the CB_1 receptor and *in vivo* pharmacology. The *in vivo* pharmacology was evaluated using the mouse model of cannabinoid behavior. 1',1'-Dimethylethyl-Δ^8-THC has K_i = 14 ± 2, but is inactive *in vivo*. The 1',1'-dimethylpropyl through 1',1'-dimethyldecyl homologues have K_i = 0.77 to 14 nM and all are full agonists *in vivo.* Although 1',1'-dimethylundecyl-Δ^8-THC has moderate affinity for the CB_1 receptor (K_i = 26 ± 6 nM), it has greatly reduced potency *in vivo*. The 1',1'-dimethyldodecyl homologue has diminished affinity for the CB_1 receptor (K_i = 126 ± 18 nM) and is effectively inactive *in vivo*.

In a QSAR study, Keimowitz *et al.* (*J. Med. Chem.* **2000**, *43*, 59) found that a side chain which could wrap around toward the cannabinoid nucleus led to enhanced potency. A similar QSAR and CoMFA analysis has been carried out on the series of 1',1'-dimethylalkyl-Δ^8-THC homologues, and it has been found that the dimethylundecyl and dimethyldodecyl side chains extend beyond the region for optimal receptor affinity and *in vivo* potency. The side chain conformations of 1',1'-dimethylpropyl through 1',1'-dimethyldecyl-Δ^8-THC are such that they can fold back in proximity to the tricyclic cannabinoid nucleus.

Acknowledgements: The work at Clemson University was supported by grant DA03590, that at Virginia Commonwealth University by grant DA03672 and that at the Research Triangle Institute by grant DA11638 all from the National Institute on Drug Abuse.

IDENTIFICATION OF AN AMINO ACID IN HELIX SIX INVOLVED WITH CB_1-LIGAND INTERACTIONS USING A COMBINED CHEMISTRY/SITE-DIRECTED MUTAGENESIS APPROACH

Robert P. Picone[1,2], Lionel A. Ayotte[1,2], Dow Hurst[4], Patricia H. Reggio[4], Atmaram D. Khanolkar[2], Donna J. Fournier[2], and Alexandros Makriyannis[1,2,3]
[1]Center for Drug Discovery, [2]Departments of Pharmaceutical Sciences, and [3]Molecular & Cell Biology, University of Connecticut, Storrs, CT 06269;
[4]Chemistry Department, Kennesaw State University, Kennesaw, GA 30144, USA

Early reports on the binding site structure of the CB_1 receptor have predominantly involved site-directed mutagenesis. A more direct method is to use covalent probes to obtain information regarding the chemical nature of the amino acids in the binding pocket. Our approach here is to use mutagenesis in tandem with covalent labeling to provide direct information on the identity of amino acids involved with ligand recognition. When combined, these methods represent a powerful and novel approach to characterize ligand binding sites. AM841, a 7´-isothiocyanato-functionalized classical cannabinoid, has been shown to bind irreversibly and with high affinity to CB_1. The electrophilic nature of the isothiocyanate and the observed irreversible binding is indicative of the presence of a nucleophilic side chain at the binding site. Interactive ligand docking studies have suggested the most likely nucleophile for the 7´-isothiocyanate was C6.47(355). The current study sought to confirm this hypothesis. This study represents the first definitive identification of an amino acid in the binding pocket of CB_1 involved in formation of a covalent bond with an affinity label.

Stably transfected human CB_1 WT and mutant CHO-K1 cell lines were prepared and evaluated for the ability to recognize [^{3}H]-CP55940, [^{3}H]-WIN55212-2, and AM841. The C6.47(355) mutations were designed to alter the nucleophilic character of the amino acid side chain at this position. The four mutations included C6.47(355) to S, A, L and K. In the case of the S, A, and L mutants, nucleophilicity was eliminated while polarity and aliphatic chain length were varied. The K mutation was included to potentially restore nucleophilic character in position 6.47(355). The data obtained indicate distinct binding domains for CP55940 and WIN55212-2. Identity of the residue in position 6.47(355) impacts the recognition of CP55940 whereas the binding of WIN55212-2 was insensitive to the identity of residue 6.47(355). Most importantly, the results obtained with AM841 in the current study are consistent with the ligand docking studies and confirm that C6.47(355) is the site of covalent bond formation for AM841.

Acknowledgements: This work has been supported by NIDA grants DA9158, DA3801, DA7215, DA3934, DA5955 and the State of Connecticut Critical Technologies Program in Drug Design

ANALOGS OF THE CB_1 ANTAGONIST SR141716 THAT SELECTIVELY DISPLACE CANNABINOID AGONISTS

Brian F. Thomas[1], Maria E. Francisco[1], Herbert H. Seltzman[1],
Anne F. Gilliam[1], Roger G. Pertwee[2], and Lesley A. Stevenson[2]
[1]Research Triangle Institute, Research Triangle Park, NC 27709, USA
[2]University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, Scotland, UK

A variety of alkyl amide analogs of SR141716A have been synthesized and tested for their ability to displace the prototypical cannabinoid ligands ([^{3}H]CP-55,940, [^{3}H]SR141716A, and [^{3}H]WIN55212-2), their ability to affect GTP-γ-[^{35}S] binding, and their effects in the mouse vas deferens. This systematic evaluation has resulted in the discovery of novel compounds with unique binding properties at the central cannabinoid receptor (CB_1), and distinctive pharmacological activities in CB_1 receptor tissue preparations. In competitive binding assays in rat brain membrane preparation, these analogs of SR141716A bind to the receptor and displace [^{3}H]CP-55,940 and [^{3}H]SR141716A much more effectively than [^{3}H]WIN55212-2. The differences in the displacement curves are much more pronounced in human brain receptor preparations than in rat brain membrane preparations, which is interesting since the amino acid sequence of each of these receptors is known, and the receptors share approximately 99% homology. This binding selectivity is not observed with SR141716A, a compound that competes approximately equally effectively with all 3 radioligands in either human or rat brain membrane preparations. In GTP-γ-[^{35}S] assays in whole rat brain, most of the SR141716A analogs possessed some inverse agonist activity. Surprisingly, when one of the compounds that failed to displace [^{3}H]WIN55212-2 as readily as it does [^{3}H]SR141716A or ^{3}H]CP-55,940 was tested in the mouse vas deferens assay, it produced little or no shift in the dose response curve for WIN55212-2. However, it possessed inverse agonist activity in the absence of the agonist (indicating that it bound to the receptor and affected cannabinoid tone). Apparently, this phenomenon is not limited to alkyl amides analogs of the aminopiperidinyl moiety, since a ring constrained analog of SR141716A (pyrazolo[1,5-f]phenanthridine ring system) also revealed selectivity in its ability to displace the radiolabeled cannabinoid agonists from the CB_1 receptor. This data provides evidence that the site of receptor interaction for WIN55212-2 is distinct from the site(s) for CP-55,940 and SR141716A.

Acknowledgement: Supported by NIDA grant DA-11638

THE TMH 3-4-5-6 AROMATIC RICH REGION OF CB_1: IMPORTANCE OF AROMATIC STACKING INTERACTIONS FOR THE BINDING OF SR141716A AND WIN55,212-2 AND C-H•••π INTERACTIONS FOR THE BINDING OF ANANDAMIDE

Patricia Reggio[1], Dow Hurst[1], Kurt Buehner[1], Judy B. Norris[1],
Sean D. McAllister[2], and Mary E. Abood[2]
[1]Chemistry Department, Kennesaw State University, Kennesaw, GA 30144
[2] Forbes Norris ALS Research Center, California Pacific Medical Center,
San Francisco, CA 94115, USA

Both the CB_1 inverse agonist, SR141716A (SR) and the CB agonist, WIN55,212-2 (WIN) are highly aromatic compounds. We hypothesized that aromatic stacking interactions might be important for the binding of these compounds at CB_1. The TMH 3-4-5-6 region is rich in aromatic residues, including F3.25(189), F3.36(200), W4.64(255), Y5.39(275), W5.43(279) and W6.48(356). Shire and co-workers have shown in CB_1/CB_2 chimera studies that the TMH-4-E2-TMH5 region of CB_1 contains residues critical for the binding of SR and WIN (*Life Sci*. ***65***: 627, **1999**). In Monte Carlo/ Stochastic Dynamics Simulations of the inactive state of WT CB_1, we saw a persistent aromatic stack between F3.36(200), W5.43(279), and W6.48(356) that appeared to be open for additional interaction with ligand. We, therefore, pursued the aromatic residue rich TMH 3-4-5-6 region as the binding site for SR and WIN. SR was docked in the TMH 3-4-5-6 region in a model of the inactive state of CB_1(R). This model used the Palczewski et al. 2.5 Å structure of rhodopsin (*Science* ***289*****:** 739, **2000**), as a starting point with modifications accounting for divergences in the location of helix bending residues such as prolines. The SR/CB_1 (R) complex was minimized using the Amber* force field in Macromodel. Results of this calculation showed that both the mono- and di-chlorophenyl rings of SR stack with W5.43(279). In addition, the dichlorophenyl ring is involved in an extended stack involving F3.36(200) and W6.48(356), while the C-3 substituent of SR is involved in a hydrogen bond with K3.28(192).

WIN in its s-trans conformation was then docked in the TMH 3-4-5-6 region in a model of the activated state of CB_1 (R*). This model reflects biophysical information which indicates that the proline kink in the CWXP motif of TMH 6 moderates and both TMHs 3 and 6 rotate during the R to R* transition in GPCRs. In the Amber* minimized WIN/CB_1 complex, the indole ring of WIN stacks with F3.36(200), while the naphthyl ring has a strong stack with W5.43(279) and a less favorable stack with W6.48(356).

Anandamide (AEA) is not an aromatic ligand. Conformational Memories (Guarnieri et al. *J. Am. Chem. Soc*. ***118***: 5580, **1996**) studies indicated that AEA can adopt a U-shaped conformation, with the hydoxyl forming an intramolecular hydrogen bond with the carboxamide oxygen. Amber* docking studies indicated that AEA binds, not in the TMH 3-4-5-6 region, but in the TMH 2-3-6-7 region. Here, the carboxamide oxygen can form a hydrogen bond with K3.28(192). The aromatic amino acid side chains of F2.57(170) and F3.25(189) form C-H •••π interactions with the C5-C6 and C11-C12 acyl chain double bonds, respectively, while other hydrophobic residues line the binding pocket.

These results suggest that the aromatic residues, F3.36(200), W5.43(279), and W6.48(356) are part of the CB_1 binding pocket for both SR and WIN, while only F3.25(189) from the TMH 3-4-5-6 aromatic rich region of CB_1 is part of the AEA binding site.

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CELLULAR LOCALIZATION OF CB_1 AND FATTY ACID AMIDE HYDROLASE mRNAs IN SELECTED AREAS OF THE HUMAN CENTRAL NERVOUS SYSTEM

Julián Romero, Rosa M. Tolón, Miguel Ángel Treviño,
Cristina Benito, Estefanía Núñez, and Alberto Rábano
Laboratorio de Apoyo a la Investigación. Fundación Hospital
Alcorcón. 28922. Alcorcón. Madrid. Spain

We have recently reported the cellular distribution of CB_1 receptors and fatty acid amide hydrolase (FAAH) in the human Central Nervous System (CNS) by immunohistochemistry. Their wide distribution and high abundancy in several brain structures point to an important role of the endocannabinoid system in different functions. Now we complete our former studies with the analysis of the pattern of expression of both receptors and enzyme by in situ hybridization. To that end, we synthesized specific digoxigenin-labelled riboprobes for CB_1 and FAAH mRNAs that allow the detection of mRNA presence at the cellular level. In situ hybridization was performed in 10 micrometers-thick cryostat sections of different human CNS structures, obtained from adult male and female with no background of neuropsychiatric disease and within a 12 hrs postmortem period. Brains were cut in small blocks that were immediately cryoprotected and frozen by immersion in liquid nitrogen. A full neuropathological examination was performed in every case on paraffin-embedded tissue, excluding any significant pathological finding. A clear cytoplasmic staining could be observed after hybridization with antisense riboprobes for CB_1 and FAAH mRNAs, incubation with an anti-digoxigenin polyclonal antibody and developing with NBT-BCIP. As expected, CB_1 receptors and FAAH enzyme are abundantly expressed in different cell´s populations, specially in frontal, parietal and motor cortices, hippocampal formation, cerebellar cortex and others. Our results confirm and extend previous findings and support a prominent role for the endocannabinoid system in motor and limbic functions.

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PHYSIOLOGICAL SIGNIFICANCE OF 2-ARACHIDONOYLGLYCEROL AS A NEUROMODULATOR

Takayuki Sugiura, Saori Oka, and Keizo Waku
Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko,
Tsukui-gun, Kanagawa 199-0195, Japan

2-Arachidonoylglycerol (2-AG) is an arachidonic acid-containing monoacylglycerol isolated from rat brain (Sugiura et al., (**1995**) Biochem. Biophys. Res. Commun. 215, 89) and canine gut (Mechoulam et al., (**1995**) Biochem. Pharmacol. 50, 83) as an endogenous cannabinoid receptor ligand. Previously, we found that 2-AG induces Ca^{2+} transients in NG108-15 cells. We confirmed that the response induced by 2-AG was blocked by pretreatment of the cells with SR141716A, indicating that the response induced by 2-AG was mediated through the CB_1 receptor (Sugiura et al., (**1996**) Biochem. Biophys. Res. Commun. 229, 58). Similar 2-AG-induced Ca^{2+} transients were observed in HL-60 cells. We confirmed that the response was abolished by pretreatment of the cells with SR144528, indicating that the response induced by 2-AG was mediated through the CB_2 receptor (Sugiura et al., (**2000**) J. Biol. Chem. 275, 605). Based on the results of structure-activity relationship experiments, we concluded that 2-AG is the endogenous natural ligand for the cannabinoid receptors (CB_1 and CB_2). In the present study, we investigated possible physiological roles of 2-AG as a neuromodulator in the brain. First, we examined the synthesis of 2-AG in rat brain synaptosomes. We found that the level of 2-AG was augmented markedly in depolarized synaptosomes. We did not detect an ether-linked analog of 2-AG (2-AG ether or noladin ether) in synaptosomes or in the brain. Treatment of synaptosomes with voltage-gated Ca^{2+} channel inhibitors partially blocked depolarization-induced generation of 2-AG, indicating that the entry of Ca^{2+} through voltage-gated Ca^{2+} channels is involved, at least in part, in the augmented synthesis of 2-AG. We also found that pretreatment of synaptosomes with U73122, a phospholipase C inhibitor, blocked depolarization-induced generation of 2-AG, suggesting that phospholipase C is involved in the generation of 2-AG. In agreement with this, we confirmed that the level of arachidonic acid-containing diacylglycerol was elevated markedly in depolarized synaptosomes. It seems possible that a large part of 2-AG was generated through combined actions of phospholipase C and diacylglycerol lipase. We found that 30 % of newly formed 2-AG was released from depolarized synaptosomes, indicating that 2-AG can act as an intercellular mediator. Next, we examined the effect of SR141716A on the release of glutamate from depolarized synaptosomes. We found that treatment of synaptosomes with SR141716A significantly enhanced the release of glutamate from synaptosomes upon depolarization. These results strongly suggest that 2-AG released from stimulated neurons may have an important physiological role in the attenuation of neurotransmission through acting on the CB_1 receptor expressed mainly in the presynapse.

IS THERE AN ANANDAMIDE TRANSPORTER?

Sherrye T. Glaser[1], Keith M. Studholme[2], Folayan Fatade[1],
Stephen Yazulla[2], Nada Abumrad[3], and Dale G. Deutsch[1]
[1]Department of Biochemistry and Cell Biology, [2]Department of Neurobiology and Behavior,
[3]Departmemt of Physiology and Biophysics, SUNY at Stony Brook, NY 11794 USA

Anandamide is an endogenous lipid compound that is taken-up by cells, and then sequestered intracellularly by membranes, or in the case of cells expressing fatty acid amide hydrolase (FAAH), it is also hydrolyzed, producing arachidonic acid and ethanolamine. While it has been proposed that anandamide uptake is a process mediated by a transporter, no transport protein has been isolated. The main evidence for a transporter comes from various groups, including ours, that observed saturable kinetics and the existence of compounds, such as AM404, which inhibited intracellular accumulation. We studied the uptake of anandamide in whole tissue and in cultured cells as described below.

Intact goldfish retinal tissue uptake studies were performed. The presence of FAAH in goldfish retina was determined by western blot and FAAH enzymatic activity which was inhibited by AM404 and MAFP with IC_{50}s of 3 μM and 20 nM, respectively. The ^{3}H-anandamide uptake pattern (as determined by emulsion autoradiography) was non-random, with the bulk of grain deposition occurring over cone photoreceptors and Mueller cells; both cell types that are FAAH-immunoreactive. A pre-incubation of the retinal tissue in either 10 μM AM404 or 1 μM MAFP resulted in a significant reduction in grain deposition ($p<0.02$ and $p<0.006$ respectively). These data showed that FAAH immunoreactive cells are responsible for most of anandamide uptake in intact tissue, and FAAH inhibitors prevent this uptake. Conversely, cells that are not FAAH immunoreactive do not accumulate anandamide.

In cultured cells, we did not observe saturable kinetics when N18TG2 neuroblastoma (which contain active FAAH) and CCF-STTG astrocytoma (which lack FAAH) were incubated in medium containing increasing concentrations of ^{3}H-anandamide under the current conditions of our assay. Furthermore, a ten minute preincubation of astrocytoma or neuroblastoma cells with 10 μM of the transport inhibitor N-(4-hydroxyphenyl)arachidonylamide (AM404) or 100 nM of the FAAH inhibitor methyl arachidonyl fluorophosphonate (MAFP), did not significantly ($p>0.2$) affect ^{3}H-anandamide accumulation. In contrast, using neuroblastoma under experimental conditions used previously by our laboratory and others, both inhibitors significantly ($p<0.01$) reduced intracellular ^{3}H-anandamide accumulation. Interestingly, all published transport inhibitors to date also inhibit FAAH and we confirm this in the current report.

In summary, these data indicate that uptake may occur by simple diffusion. Furthermore, under some conditions, compounds that inactivate FAAH affect the concentration gradient that drives anandamide into the cell so that accumulation is decreased in a manner indistinguishable from that of a postulated transport inhibitor.

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ENDOCANNABINOIDS: NOVEL MEDIATORS OF GENERAL ANESTHETIC ACTION

Cecilia J. Hillard[1], Sachin Patel[1], David J. Radamacher[1], Erica J. Carrier[1], LaToya J. Perry, Eric Wohlfiel[2], Kasem Nithipatikom, and William B. Campbell[1]
[1]Departments of Pharmacology and Toxicology, and [2]Anesthesiology
Medical College of Wisconsin, Milwaukee, WI 53226

The general anesthetic, propofol, is widely used in clinical practice, in part, due to its relative lack of adverse side effects and its rapid induction and recovery properties. Propofol, like benzodiazapine and barbiturate compounds, potentiates central GABAergic neurotransmission. Propofol affects on GABA-mediated inhibitory chloride currents certainly contribute to its anesthetic effect. However it is clear that the efficacy of general anesthetics may result from interactions with numerous protein/lipid "receptors" and that anesthesia may result from the combined effects of these drugs at multiple membrane and cytosolic sites within the brain. Here we report that endocannabinoids mediate the sleep-inducing effects of the general anesthetic, propofol. The CB_1 cannabinoid receptor antagonist, SR141716, attenuated the sleep-inducing effects of propofol *in vivo,* measured using the loss of righting reflex in mice. SR141716 had no effect on the loss of righting reflex following treatment with thiopental. *In vitro*, propofol competitively inhibited the activity of a key enzyme involved in the degradation of endocannabinoids, fatty acid amide hydrolase (FAAH), with an IC_{50} value of 3 μM. Propofol also inhibited the uptake of ^3H-anandamide into cerebellar granule cells, an assay of effects on the putative anandamide carrier (ANT), but had no effect on ligand binding to the CB_1 receptor. At the peak of the righting reflex loss, mice were sacrificed and the brains were processed and assayed for endocannabinoid concentrations. Propofol administration significantly increased the levels of the endocannabinoids anandamide and 2-arachidonyl glycerol, producing 10-15 fold increases in both compounds. We conclude that propofol administration results in enhanced brain concentrations of endocannabinoids and that this contributes significantly to the mechanism by which propofol induces anesthesia. The mechanism for the increase in endocannabinoids by propofol is not completely clear, but likely involves decreased catabolism since propofol inhibited FAAH activity and inhibited the ANT at relevant concentrations. These studies are significant for several reasons; first, they suggest that manipulation of the endocannabinoids will induce sleep and second, suggest that endocannabinoid concentrations in the brain are regulated primarily by degradation rather than synthesis. Finally, these studies suggest that one physiological role of the endocannabinoids is in the induction of natural sleep.

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FURTHER CHARACTERIZATION OF THE NOVEL ENDOCANNABINOID VIRODHAMINE

John-Michael Sauer[1], Andrea Baker Leese, Christian C. Felder, and Amy C. Porter
Eli Lilly and Company, Neuroscience Division, [1]Drug Disposition,
Lilly Corporate Center, Indianapolis, IN 46285

A novel endocannabinoid, virodhamine, was recently identified by our group during development of an LC/MS/MS assay to measure endocannabinoids in tissue and brain microdialysate. In contrast to the amide linkage of anandamide, virodhamine is arachidonic acid and ethanolamine joined by an ester linkage. Virodhamine was shown to be present in brain and peripheral tissues with higher concentrations in the periphery especially in tissues that express the CB_2 receptor. By measuring GTP-binding in membranes made from Sf9 cells overexpressing either CB_1 or CB_2 receptors virodhamine was shown to be a partial agonist at the CB_1 receptor and a full agonist at the CB_2 receptor. The present study was designed to measure the ability of virodhamine to activate native CB_1 receptors. Antibody-capture GTP-γ-^{35}S binding with an antibody specific for the Gαi subunit was used to measure the activity of virodhamine. Membranes were prepared from rat cerebellar and striatal tissue. Anandamide was used as a control to measure 100% efficacy at the CB_1 receptor. Virodhamine acted as a partial agonist in both cerebellar and striatal membranes confirming that this novel endocannabinoid acts as a partial agonist at endogenous cannabinoid receptors.

ANANDAMIDE ENHANCES EXTRACELLULAR LEVELS OF ADENOSINE AND INDUCES SLEEP: AN *IN VIVO* MICRODIALYSIS STUDY

Eric Murillo-Rodriguez[1], Carlos Blanco-Centurion[1], Dmitry Gerashchenko[1], Cristina Sanchez[2], Daniele Piomelli[2], and Priyattam J. Shiromani[1]
[1]Molecular Sleep Lab. Harvard Medical School and VA Medical Center, West Roxbury, MA. 02132. USA and [2]Department of Pharmacology, University of California, Irvine. USA

Introduction. We have investigated the role of the endocannabinoid system in sleep and found that activation of the CB_1 receptor via infusion of the endocannabinoid, anandamide (AEA), enhances sleep [4, 5]. The mechanism by which the endocannabinoids promote sleep is not known but could be linked to an induction of endogenous sleep factors. Adenosine (AD) is one such sleep factor and sleep is induced as a result of increased AD levels [7, 8]. Extracellular AD concentrations increase with prolonged waking (W) and then decline with sleep [6]. Caffeine, a non-specific AD antagonist, increases W. In the present study we investigated whether the AEA-induced sleep could be linked to an increase in AD.

Animals and surgery. Three-month-old male F344 rats (n=6) were implanted with sleep recording electrodes and a microdialysis guide cannula (IC guide, BAS) into the magnocellular area of the basal forebrain (A= -.35; L= 2.0; H= 8.5). The rats were then placed in a cage with unrestricted mobility with food and water *ad lib* and housed at constant temperature (21 ± 1°C) and under controlled light-dark cycle (lights on: 07:00 to 19:00h). One week after surgery the experiments were started.

Microdialysis sampling procedure. The microdialysis probe (BAS 1mm of length) was inserted 24h before sampling of AD levels. The following day at 07:00h, animals received DMSO (40% in saline, 1ml ip) or AEA (10mg/kg, ip). Immediately after drug or vehicle administration microdialysate samples (5µl) were collected every 20 min over 5h (flow rate= 0.25µl/min).

Adenosine analysis. AD levels were determined using HPLC. A microbore column (BAS) attached to the injector (CC-5e) and to the detector (UV-116A, BAS) achieved separation. Chromatographic data were compared to known standards (Chromgraph Report software, BAS).

Results. AEA produced a progressive increase in AD levels in the basal forebrain during the first 3h post-injection, compared to vehicle ($p<0.05$). There was a significant increase (48.56%, $p<0.05$) in total sleep time in the third hour and corresponded with peak AD levels. The increase in sleep was due primarily to an increase in slow wave sleep.

Conclusion: An interaction between the CB_1 and the AD A_1 receptor as described [1, 2, 3] could produce the increased AD found in the present study, and may be responsible for increased total sleep time.

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AGEING AND FSH REGULATE THE DEGRADATION AND PRO-APOPTOTIC ACTIVITY OF ANANDAMIDE IN MOUSE SERTOLI CELLS

Mauro Maccarrone[1], Sandra Cecconi[2], Gianna Rossi[2], Natalia Battista[1],
Monica Bari[1], and Alessandro Finazzi-Agrò[1]
[1]Department of Experimental Medicine and Biochemical Sciences, University of Rome Tor Vergata, I-00133 Rome, ITALY; [2]Department of Biomedical Sciences and Technologies, University of L'Aquila, I-67100 L'Aquila, ITALY

Anandamide (AEA) is known to adversely affect female fertility (M. Maccarrone et al. *Lancet* 355:1326, **2000**). However, a potential role of AEA in male reproductive functions is unknown. Here we report evidence that immature mouse Sertoli cells have the biochemical tools to bind and inactivate AEA, i.e. a functional type-2 cannabinoid receptor (CB2R), a selective AEA membrane transporter (AMT) and an AEA-degrading enzyme fatty acid amide hydrolase (FAAH). We show that, unlike CB2R, the activity of AMT and the activity and expression of FAAH decrease while the apoptosis-inducing activity of AEA increases with age during the neonatal period. We also show that follicle-stimulating hormone (FSH) reduces the apoptotic potential of AEA, but not that of its non-hydrolyzable analogue methanandamide. Concomitantly, FSH enhances FAAH activity, in a way dependent on mRNA transcription and protein synthesis and apparently involving cyclic AMP. These data demonstrate that Sertoli cells partake in the peripheral endocannabinoid system, and that FSH reduces the apoptotic potential of AEA by activating FAAH. Taken together, it can be suggested that the endocannabinoid network plays a role in the hormonal regulation of male fertility.

N-ACYLETHANOLAMINES POTENTIATE THE CALCIUM INFLUX RESPONSE TO ANANDAMIDE IN hVR1-HEK293 CELLS EXPRESSING VANILLOID RECEPTORS

Darren Smart[1], Kent-Olov Jonsson[2], Séverine Vandevoorde[3], Didier M. Lambert[3], and Christopher J. Fowler[2]
[1]Neuroscience Research, Glaxo SmithKline Pharmaceuticals, New Frontiers Science Park, Third Avenue, Harlow, Essex CM19 5AW, U.K., [2]Department of Pharmacology and Clinical Neuroscience, Umeå University, SE-901 87 Umeå, Sweden; and [3]Unité de Chimie pharmaceutique et de Radiopharmacie, Université catholique de Louvain, Avenue Mounier, 73, UCL-CMFA 73.40, B-1200 Brussels, Belgium

In a recent study, it was reported that the *N*-acylethanolamine palmitoylethanolamide (PEA, C16:0) potentiated the vanilloid receptor-mediated influx of Ca^{2+} into hVR1-HEK293 cells in response to anandamide (AEA, De Petrocellis *et al., FEBS Letts.* 506 [**2001**] 253-6). These authors suggested that "PEA might act as an endogenous enhancer of VR1-mediated AEA actions". In the present study, the effects of a series of PEA homologues and analogues upon the vanilloid receptor-mediated influx of Ca^{2+} into hVR1-HEK293 cells in response to AEA has been investigated using the FLIPR technique (Smart *et al.*, *Br. J. Pharmacol.* 129 [**2000**] 227-30).

Consistent with the data De Petrocellis *et al. (ibid.)* and Smart *et al. (ibid.)*, PEA (10 μM) produced a Ca^{2+} response *per se* and augmented the response to a submaximal concentration (1 μM) of AEA. Among homologues of PEA, *N*-acylethanolamines with a acyl chain length <10 carbon atoms had little or no effect, whereas the 12, 17 and 18 carbon chain length compounds produced a larger potentiation of the response to AEA than did PEA. None of the compounds (other than PEA) produced a Ca^{2+} response *per se*. The monounsaturated 18 carbon chain length compound oleoylethanolamide also greatly potentiated the Ca^{2+} response to AEA. Further experiments indicated that the 12 carbon compound (laurylethanolamide) decreased the EC_{50} values for the Ca^{2+} influx response to AEA from 1.8 to 0.22 μM as its concentration was increased from 0-10 μM. In contrast, lauroylethanolamide did not affect the dose-response sensitivity to capsaicin. In addition, there was no correlation between the ability of the compounds to inhibit rat brain AEA metabolism by FAAH and their ability to potentiate the VR1 receptor-mediated response to AEA in the hVR1-HEK293 cells. Indeed, the FAAH inhibitor palmitoyltrifluoromethyl ketone did not modulate the Ca^{2+} response to AEA in these cells.

Taken together, these data suggest that PEA is not unique in being able to potentiate the VR1 receptor-mediated response to AEA and that other *N*-acylethanolamines are indeed more efficaceous in this respect. Given that the synthesis of long chain *N*-acylethanol-amines such as palmitoyl-, stearoyl- and oleoylethanolamide are increased under conditions of cellular stress, inflammation and degeneration, it is tempting to speculate that these endogenous compounds may be important regulators of the actions of AEA upon vanilloid receptors under such conditions.

ACTIONS OF N-ARACHIDONYLGLYCINE ON INFLAMMATION AND REGULATION OF ANANDAMIDE DEGRADATION

Sumner H. Burstein[1,2], Susan M. Huang [3], Pusha Karim[1], William Pearson[1], Timothy J. Petros[3], Ronald G. Rossetti[2], J.Michael Walker[3], and Robert B. Zurier[2]
[1]Department of Biochemistry and Molecular Pharmacology, [2]Dartment of Medicine, University of Massachusetts Medical School, Worcester, MA 01 605
[3]Brown University, Departments of Psychology and Neuroscience, Providence, RI 02912

The carboxylic analog of anandamide, N-arachidonylglycine, occurs in rat and bovine brain as well as in peripheral sites and shows activity against tonic, formalin induced pain (Huang et al., **2001**; Huang et al., **2001**). It was also observed that it inhibits the hydrolytic activity of FAAH on anandamide. These and other data suggest that it may show efficacy in models of inflammation and can serve as an endogenous regulator of tissue anandamide concentrations as well. In this report, we show findings that support both of these hypotheses. Arachidonic acid induced paw edema in mice was reduced by more than 50% by the prior oral administration of 4 mg/kg of N-arachidonylglycine. *In vitro* treatment of phytohemmaglutenin stimulated T-cells with N-arachidonylglycine inhibited their proliferation at a concentration of 10 μM in contrast to anandamide that showed no effect. Treatment of RAW cells with 16 μM N-arachidonylglycine resulted in a two fold stimulation in the release of free, radiolabelled arachidonic acid suggesting activation of phospholipases. The LPS induced activation of NFκB, an important mediator of inflammation, was reduced by >75% when peripheral blood monocytes were exposed to 10 μM N-arachidonylglycine. Finally, blood levels of anandamide in rats given 10 mg/kg p.o. of N-arachidonylglycine were increased ten fold when compared to vehicle treated controls. *In vitro* evidence demonstrated that N-arachidonylglycine was not a precursor for the increased anandamide suggesting that the effect was due to the inhibition of FAAH. These findings raise the possibility that NAGly may be an endogenous regulator of FAAH activity, however, this may or may not be related to its anti-inflammatory activity.

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ISOLATION, IDENTIFICATION AND PRO-NOCICEPTIVE ACTIONS OF *N*-ARACHIDONOYL DOPAMINE (NADA), AN ENDOGENOUS MOLECULE ACTIVE AT VR1/CB_1 RECEPTORS

Susan M. Huang[1], Tiziana Bisogno[2], Timothy J. Petros[1], Jocelyn F. Krey[1], Constance J. Chu[1], Jeffrey D. Miller[3], Vincenzo Di Marzo[2], and J. Michael Walker[1]
[1]Departments of Psychology and Neuroscience, Brown University, Providence, RI 02912, USA, [2]*Endocannabinoid Research Group*, Institutes of Biomolecular Chemistry and Cybernetics, National Research Council, Pozzuoli, Napoli, Itatly,
[3]Applied Biosystems, Framingham, MA 01701, USA

N-Arachidonoyl dopamine (NADA) was first synthesized as a tool for studying the cannabinoid and vanilloid systems (Bisogno et al., *Biochem J*, **2000**; De Petrocellis et al., *FEBS Lett*, **2000**) because of its structural similarities to both endogenous cannabinoids and to capsaicin. Like anandamide, NADA binds to both CB_1 and VR1 receptors, but is more potent in activating VR1 receptors compared to anandamide. Its actions were similar to capsaicin in both VR1 transfected cells and in spinal cord slices (see Abstract by Bisogno et al., this meeting). Our interest in NADA followed the same line of reasoning as that for the discovery of endogenous N-arachidonyl glycine (NAGly) (Huang et al., **2001**). The initial hypothesis of NAGly's existence was based on the structure of the molecule consisting of arachidonic acid conjugated to glycine. NADA is akin to NAGly in that it also consists of arachidonic acid attached to a small molecule (dopamine) via an amide bond. We therefore hypothesized that NADA maybe a naturally occurring molecule in mammalian nervous system.

Striata from fresh bovine brain were extracted via liquid-liquid and solid-phase extractions, and NADA was identified via mass spectrometry. We analyzed the levels of NADA in the rat nervous system. Of various parts of the brain analyzed, the striatum and the hippocampus contained the highest level of NADA. NADA was also present in the peripheral nervous system, evidenced by its detection in bovine dorsal root ganglion.

One consequence of activation of VR1 receptors is induction of pain. Hence we tested NADA's ability to elicit pain behavior in rats. Peripheral administration of NADA in the hindpaws of rats dose-dependently (0.1-10 μg) caused hyperalgesia to thermal stimuli. The effect is blocked by the VR1 antagonists capsazepine and iodo-resiniferatoxin.

As NADA binds to both CB_1 and VR1 receptors, its isolation could suggest a possible role as an endocannabinoid or endovanilloid. However, NADA seemed to be acting as a vanilloid agonist, at least in the periphery, to produce pain. Hence these findings support the notion that NADA is a naturally occurring molecule in mammals that may serve as an endogenous ligand for VR1 receptors. The circumstances under which it acts at the CB_1 receptors remain to be determined.

AN ENDOGENOUS CAPSAICIN-LIKE COMPOUND THAT ACTIVATES BOTH VANILLOID AND CANNABINOID RECEPTORS

Tiziana Bisogno[1], Susan M. Huang[2], Marcello Trevisani[3], Abdulmonem Al-Hayani[4], Luciano De Petrocellis[1], Filomena Fezza[1], Michele Tognetto[3] Stephen N. Davies[4], Pierangelo Geppetti[3], J. Michael Walker[2], and Vincenzo Di Marzo[1]
[1,2]Endocannabinoid Research Group, Institutes of [1]Biomolecular Chemistry and [2]Cybernetics, C.N.R., Pozzuoli (NA), Italy,
[2]Departments of Psychology and Neuroscience, Brown University, Providence, RI 02912 USA,
[3]Department of Experimental and Clinical Medicine, University of Ferrara, Ferrara, Italy, [4]Department of Biomedical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen, UK

The vanilloid (VR1) receptor is a non-selective cation channel that is most abundant in peripheral sensory fibers, but is also found in several brain nuclei. VR1 is gated by protons, heat and the pungent ingredient of 'hot' chili peppers, capsaicin (Szallasi and Blumberg, *Pharmacol. Rev*., **1999**). Recently, it has been shown that the endocannabinoid anandamide acts as a full agonist at VR1 receptors (Zygmunt et al., *Nature*, **1999**). Although its potency at VR1 is rather low, the vanilloid-like activity of anandamide can be enhanced by several regulatory factors (Di Marzo et al., *Trends Pharmacol. Sci*., **2001**). To date, however, no endogenous compound with potency at this receptor comparable to that of capsaicin has been identified. Recently we reported the chemical synthesis and cannabimimetic pharmacological activity of *N*-arachidonoyl-dopamine (NADA), an anandamide analogue capable of activating the cannabinoid CB_1 receptor (Bisogno et al., *Biochem. J.*, **2000**). Here we examined the hypothesis, based on previous structure activity relationship studies and on the availability of biosynthetic precursors, that NADA is also an endogenous potent agonist of VR1 receptors in mammalian nervous tissues.

By using mass spectrometric techniques (see Abstract by Huang et al., at this meeting), NADA was detected in both central and peripheral nervous tissues from the rat, and several possible routes were found for its biosynthesis and inactivation in rat brain. NADA activates VR1-mediated Ca^{2+} influx into HEK293 cells over-expressing either human or rat VR1 with a potency (EC_{50}~50 nM) and efficacy similar to those of capsaicin. Furthermore, NADA potently activates native VR1 receptors in neurons from rat dorsal root ganglion by inducing Ca^{2+} influx in these neurons (EC_{50}~750 nM), and the release of substance P and CGRP from spinal cord slices (EC_{50}~100 nM). NADA potently activates also native VR1 receptors in hippocampus slices, thereby enhancing hippocampal paired-pulse depression (EC_{50}~200 nM).

This is the first demonstration of the existence of a capsaicin-like substance in mammals with a possible role in the modulation of hippocampal synaptic plasticity and in the generation of hyperalgesia. Together with our previous finding of the functional activity of NADA on CB_1 receptors, these data support our hypothesis that vanilloid VR1 and cannabinoid CB_1 receptors are "ionotropic" and "metabotropic" receptors, respectively, for the same class of arachidonate-derived mediators including, to date, anandamide (more potent at CB_1) and NADA (more potent at VR1).

MEMBRANE TRAFFICKING OF OPIOID RECEPTORS IN NEURAL CELLS

M. von Zastrow, K. Kim, H. Haberstock-Debic, M. Wein,
R. Neve, M. Barrot, E. Nestle, and A. Svingos
Departments of Psychiatry and Cellular and Molecular Pharmacology, University of California, San Francisco, CA; Department of Psychiatry and Center for Basic Neuroscience, UT Southwestern Medical Center, Dallas, TX; Department of Neurology, Weill Medical College of Cornell University, New York, NY, USA

Membrane trafficking processes are thought to play a fundamental role in regulating CNS opioid receptors in response to physiological and pharmacological stimuli. However cellular and molecular mechanisms that mediate opioid receptor trafficking have been studied primarily using non-neural model systems. We have utilized several approaches to investigate regulated membrane trafficking of opioid receptors in neurosecretory cells and neurons. While certain aspects of opioid receptor membrane trafficking are similar in neural and non-neural cell types, our studies suggest the existence of two additional features of opioid receptor trafficking which are observed specifically in neurons. First, in contrast to their constitutive targeting to the plasma membrane in non-neural cells, recently synthesized delta opioid receptors (DOR) are sorted from the trans-Golgi network to a regulated export pathway in neurosecretory cells and neurons. Studies of the underlying molecular mechanism define a novel membrane sorting signal in the cytoplasmic tail of DOR and identify an unanticipated role of receptor tyrosine kinase signaling in regulating anterograde membrane trafficking of opioid receptors. Second, we have observed an unanticipated process of regulated membrane trafficking of MOR occurring selectively in dendrites of CNS neurons. This process regulates both recombinant and endogenously expressed MOR in nucleus accumbens neurons *in vivo* and may play an important role in mediating physiological adaptation of CNS neurons to opiate drugs such as morphine.

CHRONIC DRUG EFFECTS ON OPIOID AND CANNABINOID RECEPTOR ACTIVATION OF G-PROTEINS IN BRAIN

S. Childers
Department of Physiology/Pharmacology, Wake Forest University
School of Medicine, Winston-Salem, NC

Mu opioid receptor and CB_1 cannabinoid receptors both belong to the same family of G-protein-coupled receptors that activate $G_{i/o}$ proteins. In most cases, chronic agonist exposure produces a varying degree of receptor/G-protein uncoupling and receptor downregulation, but the relationship between these actions and development of opioid and cannabinoid tolerance remains unclear. Chronic treatment of rats with THC produces profound decrease in CB_1 activation of G-proteins, with the degree of uncoupling varying between regions. This uncoupling is accompanied by significant decreases in CB_1 receptor number, occurring with a time course later than that of the G-protein uncoupling. Chronic treatment of rats with mu agonists (morphine, heroin, or methadone) also produces a region-selective decrease in receptor/G-protein uncoupling, but no accompanying decrease in mu receptor number. The time course of mu receptor uncoupling varies across different brain regions, with brainstem areas uncoupling first, followed by midbrain and finally forebrain. This uncoupling is reversible, with mu receptor-G-protein uncoupling returning to normal levels before the disappearance of withdrawal symptoms, and in regions in the reverse order of the onset of uncoupling. These studies reveal numerous similarities and differences in the ways in which CB_1 and mu receptors respond to chronic agonist exposure in brain.

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CELLULAR BASIS FOR STRIATAL CANNABINOID-OPIOID INTERACTIONS

V.M. Pickel[1], J. Rodrígues[1], J. Chan[1], and K. Mackie[2]
[1]Department of Neurology and Neuroscience, Weill Medical College of Cornell University, New York, NY 10021
[2]Department of Anesthesiology, University of Washington, Seattle, Washington 98195

Activation of cannabinoid subtype 1 (CB_1) receptors as well as deletions of the CB_1 gene in CB_1 (-/-) mice produce hypolocomotion, and catalepsy. Opiates active at mu-opioid receptors (MOR) can also produce similar behavioral responses through mechanisms involving the caudate-putamen nucleus (CPN) or nucleus accumbens shell (NAcSH), respectively. We used electron microscopic immunolabeling of CB_1 and MOR to show that in normal rats and mice, spiny neurons in each region show dendritic plasma membrane labeling of both receptors. More often, however, the CB_1 receptor was located on excitatory-type terminals presynaptic to spines containing MOR. In the NAcSH of CB_1 (-/-) mice, the plasma membrane density of MOR immunogold was significantly less than in wild-type controls. Together, the results suggest that cannabinoids and opiates dually modulate the output of single striatal neurons, and that chronic absence of CB_1 receptors can decrease the availability of MOR in the targeted neurons.

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OPIOID-CANNABINOID CROSS TALK: A ROLE FOR RECEPTOR-RECEPTOR INTERACTIONS

C. Rios, S. Haberny, I. Gomes, N. Trapaidze, and L.A. Devi
Department of Pharmacology, New York University School of Medicine,
New York, NY, USA

A number of previous studies have reported functional interactions between opioid and cannabinoid receptors. However, molecular mechanisms for this phenomenon have not been well explored. We addressed this by examining if opioid and cannabinoid receptors physically interact with each other and if these interactions modulate their function. We find that, when co-expressed in heterologous cells, mu, delta and kappa opioid receptors physically interact with CB_1 cannabinoid receptors. We examined the functional implications of these interactions in rat striatum- a tissue that has been reported to express all of these receptors. The examination of the effect of individual or a combination of ligands on [^{35}S]GTPgammaS binding showed that the dose-dependent increase in [^{35}S]GTPgammaS binding by the cannabinoid receptor agonist WIN-2 is augmented in the presence of the kappa selective agonist or antagonist. Interestingly, WIN-2 mediated [^{35}S]GTPgammaS binding is significantly decreased by the mu receptor agonist, DAMGO. These results suggest that interactions between opioid and cannabinoid receptors differentially affect their function. In order to further characterize the interactions between mu opioid and CB_1 cannabinoid receptors we have initiated studies in F-11 cells; a cell line that endogenously expresses these receptors. Our results examining the level of mitogen activated protein kinase phosphorylation in response to opiates in the presence of CB_1 receptor ligands are consistent with the proposal that interactions between these receptors differentially regulate their activity.

DETECTION OF ORL_1 DIMERIZATION IN LIVING CELLS USING FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET)

M. Corbani, L. Puntil, A. Lopez, C. Gonindard, F.E. L'Faqihi-Olive, and J.C. Meunier
Institut de Pharmacologie et de Biologie Structurale, CNRS, 31077 Toulouse, France

Recently, several GPCRs, including opiate receptors, which were supposed to be monomeric proteins, have been shown to co-immunoprecipitate and appeared as multimers on SDS-PAGE analysis. The multimer state seemed regulated in the presence of ligands. In order to address the question of ORL1 oligomerization in living cells, we expressed in HEK 293 cells ORL1 carrying at the C terminus one molecule of EBFP (as fluorescence donor) or one EGFP (as acceptor). Both ORL1-EBFP and ORL1-EGFP were expressed in a 1:1 ratio and clones expressing increasing levels of receptors were selected. The receptor oligomerization was monitored by FRET by recording the fluorescence emission of the cell surface after laser excitation at 360 nm using a microspectrofluorimeter. For the cells expressing ORL1 up to 5 picomoles/mg of protein, the addition of 10^{-7} M of nociceptin (Noc) induced, in 56% of the cells, a shift of 30% of the fluorescence from 460 nm (blue) to 520 nm (green). The other cells showed no shift (27%), or a reverse shift (17%), while untransfected cells and cells expressing only ORL1-EBFP or only ORL1-EGFP showed no transfer. No FRET was observed at 4°C whereas at 22°C, the kinetics showed an increasing FRET with a maximal at 2 min. The non peptide agonist Lofentanil (10^{-6} M) induced ORL1 oligomerization as well. For the clones expressing between 6 and 16 picomoles of ORL1/mg protein, a spontaneous, expression-dependent emission at 520 nm was detected in the absence of agonist, indicating vicinity of the receptors. Application of Noc induced for few cells (20%) some additional transfer, suggesting that ORL1 was probably already dimerized at these receptor densities. Our results suggest that agonist binding induces ORL1 dimerization, the agonist effect being less efficient when receptor density increases.

TIME COURSE FOR NORMALIZATION OF CB_1 RECEPTOR LEVELS AND G-PROTEIN ACTIVATION FOLLOWING CESSATION OF CHRONIC CANNABINOID TREATMENT

Laura J. Sim-Selley, W. Kirk Rorrer, George D. Dalton, Billy R. Martin, and Dana E. Selley
Department of Pharmacology and Toxicology and Institute for Drug and Alcohol Studies, Virginia Commonwealth University Medical College of Virginia, Richmond, VA 23298

Previous studies showed that chronic administration of Δ^9-THC or WIN55,212-2 produced CB_1 receptor downregulation and desensitization and in vivo tolerance. Furthermore, the magnitude and rate of development of cellular effects varied among brain regions. Region-specific effects may be reflected in vivo because studies have shown that the rate of recovery from tolerance to hypoactivity was faster than tolerance to antinociception. Therefore, these studies were conducted to determine the time course for normalization of CB_1 receptor binding and G-protein activation in different regions following cessation of cannabinoid treatment. Mice were injected twice daily (s.c.) with Δ^9-THC or WIN55,212-2 at initial doses of 10 mg/kg and 3 mg/kg, respectively. These doses were doubled every three days to final doses of 160 mg/kg for Δ^9-THC and 48 mg/kg WIN55,212-2 at day 15. Brains were collected at 1, 3, 7 and 14 days after the final injection and dissected into cerebellum, striatum and hippocampus. Decreased cannabinoid-stimulated [^{35}S]GTPγS binding was found in membrane homogenates from all regions at the 1-day point, but the reduction in cerebellum was small and this region was not analyzed further. The E_{max} for cannabinoid-stimulated [^{35}S]GTPγS binding in vehicle-treated mice was 98% in striatum and 150% in hippocampus. Cannabinoid-stimulated [^{35}S]GTPγS binding in the striatum at day 1 was approximately 70% of vehicle control Δ^9-THC and WIN55,212-20-treate mice. By day 3, percent control binding was 95% in WIN55,212-2 and 88% in Δ^9-THC-treated mice, which did not significantly differ from vehicle. In the hippocampus, percent control binding in the WIN55,212-2-treated mice was 61% on day 1 and did not return to vehicle levels until day 14 where it was 89% of control. Similarly, percent control binding in Δ^9-THC-treated mice was 53% of control on day 1 and returned to 83% of vehicle control at day 14, which did not significantly differ from vehicle. No significant changes in the levels of $G\alpha_i$ or $G\alpha_o$ were found in these regions, suggesting that decreases in cannabinoid-stimulated [^{35}S]GTPγS binding resulted from receptor uncoupling or loss of binding sites. These data demonstrate that cannabinoid receptor desensitization persists for 3-14 days following cessation of treatment and that the persistence of desensitization varies among regions. These findings suggest that different factors may contribute to CB_1 receptor desensitization in different brain regions. Furthermore, regional differences in the persistence of desensitization may produce differences in the time course for recovery from tolerance to cannabinoid-mediated effects in vivo.

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DEPRESSION OF GABAERGIC NEUROTRANSMISSION BY CANNABINOIDS IN THE VENTRAL TEGMENTAL AREA

Bela Szabo, Sabine Siemes, and Ilka Wallmichrath
Institut für Pharmakologie, Albert-Ludwigs-Universität, 79104 Freiburg, Germany

It was shown recently that Δ^9-tetrahydrocannabinol, like many other drugs eliciting euphoria, increases dopamine release in the nucleus accumbens of conscious rats (Tanda et al., Science 276:2048-2050, **1997**). Since cannabinoids have no direct action on terminals of dopaminergic neurons in the nucleus accumbens (Szabo et al., J Neurochem 73:1084-1089, **1999**), they probably increase their firing rate. The hypothesis of the present work was that cannabinoids depress GABAergic inhibition of dopaminergic neurons in the VTA which project to the nucleus accumbens.

Electrophysiological properties of VTA neurons in coronal rat midbrain slices were studied with the patch-clamp technique. The $GABA_A$ receptor antagonist bicuculline (20 µM) did not change the firing rate and the holding current of the neurons, indicating absence of a GABAergic inhibitory tone in our slice preparation. $GABA_A$ receptor-mediated inhibitory postsynaptic currents (IPSCs) were evoked by electrical stimulation in the vicinity of the recorded neurons. The amplitude of IPSCs was depressed by the synthetic mixed CB_1/CB_2 cannabinoid receptor agonist (+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)-methyl]-pyrrolo[1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl)-methanone mesylate (WIN55212-2; 1 and 10 µM) (Fig. 1). The CB_1-selective antagonist N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-3-pyrazole-carboxamide (SR141716A; 1 µM) abolished the inhibition produced by WIN55212-2 (10 µM) (Fig. 1). Two kinds of experiments were carried out to determine whether IPSCs were depressed with a pre- or postsynaptic mechanism. Currents evoked by ejection of muscimol from a pipette in the vicinity of the recorded neurons were only slightly changed by WIN55212-2 (10 µM). The frequency and amplitude of miniature IPSCs recorded in the presence of tetrodotoxin were also not changed by WIN55212-2 (10 µM).

The results indicate that activation of CB_1 cannabinoid receptors inhibits GABAergic neurotransmission in the VTA with a presynaptic mechanism. Depression of the GABAergic inhibitory input of dopaminergic neurons would increase their firing rate in vivo. Accordingly, dopamine release in the projection region of VTA neurons, the nucleus accumbens, would also increase.

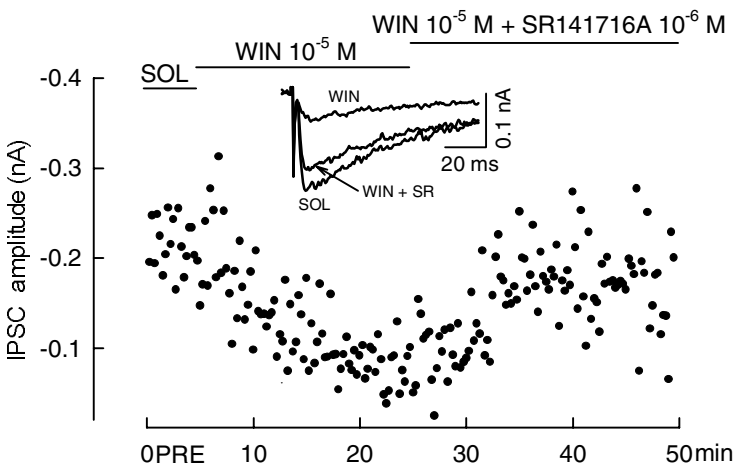

Fig. 1. Effects of solvent (SOL), WIN55212-2 (WIN) and SR141716A (SR) on inhibitory postsynaptic currents (IPSCs). IPSCs were evoked every 15 s by single electrical pulses. The inset shows averaged IPSCs obtained during superfusion with SOL, WIN 10^{-5} M and SR 10^{-6} M + WIN 10^{-5} M.

MAP KINASES PATHWAY PLAYS A ROLE IN CANNABINOID TOLERANCE

Tiziana Rubino, Daniela Viganò[1], Renata Zippel[2], and Daniela Parolaro
DBSF, Pharmacology Unit, University of Insubria, Busto Arsizio (VA),
[1]Department of Pharmacology, University of Milan, Milan
[2]Department of General Physiology and Biochemistry, University of Milan, Milan, Italy

Cannabinoids exert most of their psychoactive effects through the CB_1 receptor. This G protein-coupled receptor has been shown to be functionally coupled to inhibition of adenylyl ciclase and modulation of calcium and potassium channels. Moreover in non-neuronal cells, stimulation of transfected CB_1 receptor leads to activation of MAP kinases of the ERK subfamily, as well as JNK. Furthermore in hippocampal slices, stimulation of CB_1 receptor activates p38 MAPK.However intraneuronal signaling events mediated by CB_1 receptor stimulation in the brain in vivo remain to be established.

Starting from these considerations, the present work was organized to evaluate the cellular alteration in the MAP kinase cascade induced by in vivo THC in specific brain regions measuring the level of phosphorilated ERK1/ERK2, JNK and p38 MAP kinases in rats acutely or chronically treated with THC. For the acute studies animals were injected with THC at the dose of 15 mg/kg, ip, while for the chronic ones THC (15 mg/kg, ip) was administered in rats twice a day for 6.5 days. Thirty minutes after the last injection, brains were quickly removed and the cerebral areas for western blot analysis were obtained by gross dissection on ice.

THC acutely injected in rats did not significantly affect the phosphorilation level of MAP kinases, while chronic THC activated (phosphorilated) ERK1/ERK2 signalling in specific brain areas, with the higher increases in the areas showing the densiest CB_1 receptor content such as the striatum and the cerebellum.

In order to confirm the involvement of MAP kinases signalling in the chronic effects of THC we evaluated the acute and chronic effects of THC in mice lacking the ras-GRF1 gene. The exchange factor ras-GRF1 activates ras proteins that represent the molecular switch to trigger the sequential activation of the members of MAP kinase cascade. Acute injection of THC (10 mg/kg s.c) did not alter in ko mice the occurrence of two classical cannabinoid effects such as analgesia and hypolocomotion. On the contrary in mice lacking ras-GRF1, chronic injection of THC (10mg/kg s.c. twice a day for 5 days) did not develop tolerance to the analgesic and hypomotility effects.

Our results support the role of this signalling pathways in some biochemical and behavioural responses related to the development of cannabinoid addiction.

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CB_1 RECEPTOR-DEPENDENT MODULATION OF SEIZURE FREQUENCY AND DURATION IN EPILEPTIC RATS: IMPLICATIONS FOR THE ENDOCANNABINOID SYSTEM IN THE TREATMENT OF EPILEPSY

Melisa J. Wallace[1,2], Robert E. Blair[1], Batool Razvi[1],
Billy R Martin[2] and Robert J. DeLorenzo[1,2]
Departments of Neurology[1] and Pharmacology and Toxicology[2], Virginia Commonwealth University, Richmond, Virginia 23298, USA

Studies carried out in our laboratory have demonstrated that cannabinoid and cannabimimetic compounds exhibit anticonvulsant activity through activation of the CB_1 receptor when evaluated in the mouse maximal electroshock seizure model {Eur. J. Pharm, **2001** 428(1) 51-7}. In the present study, the pilocarpine preparation of temporal lobe epilepsy in the rat was utilized to evaluate the role of the endocannabinoid system in modulating seizure activity in an *in vivo* model of temporal lobe epilepsy. This model of epilepsy in the rat has been shown to manifest both the clinical and pathophysiological changes seen in human temporal lobe epilepsy. Epileptic animals exhibited spontaneous recurrent seizures (SRSs) evidenced by both behavioral and brain electroencephalographic (EEG) characteristics that persisted for the life of the animal. This model is ideally suited to study the effects of CB_1 receptor agonist and antagonists on spontaneously occurring seizure activity.

Eight to ten months after the induction of epilepsy, we modulated CB_1 receptor activity by administering agonists and antagonists and measured changes in seizure expression via continuous video monitoring of behavioral seizures and continuous EEG recordings from scalp electrodes of electrographic seizures. Administration of the CB_1 agonist (+) WIN55,212-3 blocked the expression of spontaneous seizure activity, demonstrating the anticonvulsant effect of CB_1 receptor activation. The inactive isomer (-) WIN55,212-3 (5 mg/kg i.p.), as well as vehicle control (Emulphor:ethanol:saline, 1:1:18) had no effect on seizure expression. In contrast, antagonism of the CB_1 receptor in the intact animal by administration of SR141716A significantly increased both seizure frequency and duration. These results demonstrate that inhibiting endogenous CB_1 receptor activation dramatically increased seizure activity.

The results of this investigation confirm the anticonvulsant properties of CB_1 receptor activation in an intact animal model of epilepsy. These data suggest a role for the endocannibinoid system in regulating the neuronal hyperexcitability associated with this model of temporal lobe epilepsy. Therefore, activation of the CB_1 receptor may represent a novel mechanism for regulating seizure activity and neuronal excitability. Understanding the effect of CB_1 activation may provide novel therapeutic insights into the treatment of epilepsy.

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EFFECTS OF THE CB_1 RECEPTOR ANTAGONIST AM251 IN INDUCED-SEIZURE IN RODENTS

Tung M. Fong[1], Wayne Rycroft[2], Lex H.T. Van der Ploeg[1], and Peter H. Hutson[2]
[1]Merck Research Laboratories, Rahway, NJ 07065, USA
[2]Merck Sharp and Dohme Neuroscience Research Center, Terlings Park, Harlow, Essex, CM20 2QR, UK

It has been reported that CB_1 receptor knockout mice exhibit increased mortality that is apparently dependent on the genetic background of the mice. However it is uncertain if CB_1 receptor antagonists would produce similar effect as the gene knockout. AM251 [(N-piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide] is a CB_1 receptor inverse agonist in vitro (binding IC50 = 3 nM). In the pentylenetetrazole (PTZ) and electroconvulsive shock (ECS) proconvulsant models, AM251 at a dose of 30mg/kg p.o. potentiated clonic seizures in both models and significantly ($p < 0.01$) reduced the dose of PTZ required to produce a clonic convulsion. Thus in vehicle pretreated animals, PTZ caused clonic seizures at a dose of 30 ± 0.8 mg/kg i.v.; whereas in mice pretreated with AM251 (30 mg/kg p.o.) the dose of PTZ producing clonic seizures was 26 ± 0.6 mg/kg i.v. In the ECS model at a current of 10mA 10% of vehicle treated animals showed clonic seizures however in mice pretreated with AM251 70% of mice developed clonic seizures. The potentiation of clonic seizures in mice in two different models of convulsant activity warrants further analysis of the role of CB_1 receptor antagonists in selected seizure models.

EVALUATION OF THE ENZYMATIC STABILITY AND THE INTRAOCULAR PRESSURE EFFECTS OF 2-AG AND NOLADIN ETHER

Krista Laine[1,3], Kristiina Järvinen[2], Raphael Mechoulam[3],
Aviva Breuer[3], and Tomi Järvinen[1]
[1]Department of Pharmaceutical Chemistry, University of Kuopio, Kuopio, Finland
[2]Department of Pharmaceutics, University of Kuopio, Kuopio, Finland
[3]Department of Medicinal Chemistry and Natural Products, Hebrew University, Israel

The endogenous cannabinoids *N*-arachidonylethanolamide (AEA) and 2-arachidonylglycerol (2-AG) are known to decrease intraocular pressure (IOP) in rabbits (Pate et al., Curr. Eye Res.14: 791-797, **1995**; Pate et al., Pharm. Res. 14: 1738-1743, **1997**). Recently, a novel putative endogenous CB_1 receptor ligand, noladin ether, was isolated from porcine brain (Hanuš et al., Proc. Natl. Acad. Sci. 98: 3662-3665, **2001**). In this study, the rates of degradation for AEA, 2-AG and noladin ether were determined in ocular tissues, and the IOP effects of topically administered 2-AG and noladin ether were compared.

The degradation of 2-AG, AEA and noladin ether was determined in 10 % bovine iris-ciliary body and cornea homogenates. In order to prepare eye drop solutions, 2-AG and noladin ether were dissolved in either hydroxypropyl-β-cyclodextrin (HP-β-CD) or propylene glycol, and administered unilaterally to the normotensive rabbit eye. IOPs of treated and untreated eyes were measured at fixed time intervals. The CB_1 receptor antagonist AM251, dissolved in HP-β-CD, was ocularly administered prior to the administration of cannabinoids to investigate if CB_1 receptors mediate the IOP effects produced by 2-AG and noladin ether.

Noladin ether ($t½$ = 430.0 ± 0.0 min, n = 2) hydrolysed at a slower rate than either 2-AG ($t½$ = 3.1 ± 0.9 min, n = 3) or AEA ($t½$ = 8.7 ± 3.8 min, n = 3) in 10 % bovine iris-ciliary body homogenate. The corresponding half-lives in 10 % bovine cornea homogenate were 1128.7 ± 532.1 min (n = 2) for noladin ether, 19.1 ± 2.6 min (n = 3) for 2-AG and 178.7 ± 21.2 min (n = 3) for AEA, respectively. The main degradation product for 2-AG and AEA eluted at the same retention time as a synthetic arachidonic acid standard.

The IOP effects of 2-AG were bi-phasic at the doses of 31.25 - 125 µg; *i.e.* initial IOP hypertension followed by IOP reduction were observed. Noladin ether, at the dose-range of 15.63 - 125 µg, reduced IOP directly after its ocular administration with no initial IOP increase in the treated eye. The CB_1 receptor antagonist AM251 (dose = 25 µg) inhibited the IOP effects of noladin ether, but did not affect those of 2-AG. Neither 2-AG nor noladin ether treatments had a significant effect on the IOP of untreated eyes.

In conclusion, the results demonstrate that the topical administration of noladin ether decreases IOP significantly in rabbits. The IOP effects of noladin ether are most probably mediated through the ocular CB_1 receptor, whereas the IOP effects of 2-AG are mediated by its arachidonic acid metabolites. This data also suggest that noladin ether does not form arachidonic acid in the eye after its topical administration.

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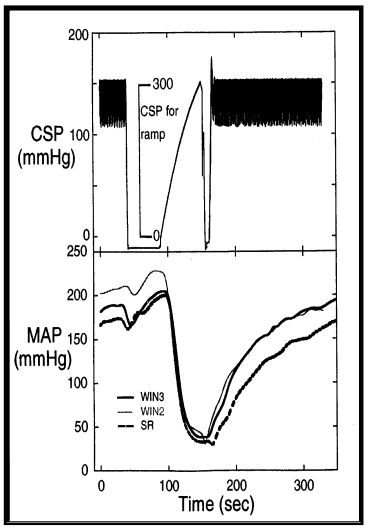
CANNABINOID MICROINJECTION INTO THE NUCLEUS TRACTUS SOLITARIUS AFFECTS BARORECEPTOR-MEDIATED CONTROL OF DYNAMIC ARTERIAL BLOOD PRESSURE CHANGES

David J. Rademacher[1], Sachin Patel[1], Caron Dean[2], Francis A. Hopp[2], Cecilia J. Hillard[1], and Jeanne L. Seagard[2]
[1] Department of Pharmacology and Toxicology, [2] Zablocki Department of Veterans Affairs Medical Center, Medical College of Wisconsin, Milwaukee, WI, USA

Fig. 1. Effects of microinjection of CB_1 receptor agonists and antagonists into the NTS on the baroreflex-induced decrease in mean arterial pressure (MAP) in response to ramp changes in CSP.

Activation of arterial baroreceptors by stretch due to increases in blood pressure (BP) in receptive areas produces a reflex depressor and bradycardiac response. The first site of CNS termination of both type I carotid sinus baroreceptors afferents, which regulate dynamic changes in BP, and type II carotid sinus baroreceptor afferents, which regulate resting, baseline BP (Seagard *et al., Circ. Res.* **1993**, *72,* 1011) are in the nucleus tractus solitarius (NTS) (Ruiz-Pesini *et al., Brain Res. Bull.* **1995**, *37,* 41). Glutamate is the primary baroreceptor neurotransmitter within the NTS leading to both NMDA- and non-NMDA-mediated activation of NTS baroreceptor-sensitive neurons (Seagard *et al., Neurosci. Lett.,* **1995**, *191,* 13). Based on evidence of CB_1 receptor expression in the NTS (Tsou *et al., Neurosci.* **1998**, *83,* 393) and the finding that central administration of cannabinoids affected BP and heart rate (Neiderhoffer, *J. Pharmacol. Exp. Ther.* **2000**, *294,* 707) we hypothesized that microinjection of CB_1 receptor agonists and antagonists into the NTS would modulate the baroreceptor reflex. As illustrated in Fig. 1, microinjection of the CB_1 receptor agonist, WIN55212-2 (WIN2) (5 μM), its inactive enantiomer, WIN55212-3 (WIN3) (5 μM), and the CB_1 receptor antagonist, SR141716A (SR) (10 μM), had little effect on the baroreflex-induced decrease in mean arterial pressure (MAP) in response to the ramp increase in carotid sinus pressure (CSP). Microinjection of WIN55212-2 increased baseline MAP during constant CSP perfusion relative to the WIN55212-3 control. The most robust effect was the delay in recovery to baseline MAP after the ramp increase in CSP as a result of microinjection of SR141716A. We hypothesize that ramp increases in CSP induce NTS barosensitive neurons to synthesize endocannabinoids which act as retrograde chemical messengers which bind to CB_1 receptors presynaptically leading in a decrease in glutamate release from the sensory neuron. SR141716A blocks presynaptic CB_1 receptors and NTS barosensitive neurons are overstimulated which results in an enhanced inhibition of sympathetic outflow. This overstimulation does not reveal itself as a change in the peak decrease in MAP but instead as a delay in the recovery of MAP after the stimulus is removed due to depressed sympathetic activity.



ENDOCANNABINOID MECHANISMS OF STRESS-INDUCED ANALGESIA

Andrea G. Hohmann, Mark H. Neely, Richard L. Suplita III, Andrea G. Nackley,
Jesse Farthing, Philip V. Holmes, and Jonathon D. Crystal
Biopsychology Program, Department of Psychology,
The University of Georgia, Athens, GA, USA

A large body of literature indicates that environmental stressors activate an endogenous mechanism for suppressing pain that is insensitive to opioid antagonists. However, the mechanism underlying nonopioid stress-induced analgesia has remained a major gap in the knowledge base. The present studies were conducted to test the hypothesis that nonopioid stress-induced analgesia is mediated by a cannabinoid mechanism. Nonopioid stress-induced analgesia was induced in rats using brief, continuous footshock. SR141716A, a competitive antagonist for cannabinoid CB_1 receptors, blocked nonopioid stress-induced analgesia, defined behaviorally in the tail-flick test. By contrast, the opioid antagonist naltrexone and the competitive antagonist for cannabinoid CB_2 receptors, SR144528, failed to alter stress analgesia in this paradigm. Blocking reuptake of the endocannabinoid anandamide with AM404 potentiated nonopioid stress-induced analgesia. Acute administration of the prototypic classical cannabinoid Δ^9-tetrahydrocannabinol similarly enhanced stress analgesia. Stress analgesia was attenuated in rats rendered tolerant to the potent cannabinoid agonist WIN55,212-2 but not in rats rendered tolerant to morphine. These data are consistent with direct support for the hypothesis that environmental stressors release endocannabinoids, as demonstrated by high performance liquid chromatography mass spectrometry (HPLC/MS). Administration of SR141716A directly to the dorsal periaqueductal gray or rostral ventral medulla attenuated nonopioid stress analgesia, suggesting that a descending cannabinoid modulatory system is activated by stress to produce adaptive changes in pain responsiveness. The present data demonstrate that nonopioid stress-induced analgesia proceeds through a CB_1 mechanism and is mediated by endogenous cannabinoids.

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CENTRAL SITE OF ACTION FOR CANNABINOIDS AS ANTI-EMETICS

Marja Van Sickle, Lorraine Oland, Ken Mackie[1], Joseph Davison, and Keith Sharkey
Neuroscience and Gastrointestinal Research Groups, Department of Physiology and Biophysics, University of Calgary, Calgary, AB T2N 4N1, Canada and,
[1]Departments of Anesthesiology, Physiology and Biophysics,
University of Washington, Seattle, WA 98195-6540, USA

The anti-emetic properties of cannabinoids have been shown in animal models and in man. Common sites of action for anti-emetic drugs include the terminals of vagal afferent nerves in the upper gastrointestinal tract and brainstem, as well as the dorsal vagal complex in the brainstem. The cannabinoid 1 receptor (CB1R) is present in the enteric nervous system and in the dorsal vagal complex. Within the brainstem, CB1R are not on vagal afferent terminals. Vagal afferent terminals in the stomach and duodenum are a possible site of anti-emetic action of cannabinoids, but have not been examined. The aim of this study was to localize the site of action of cannabinoids peripherally or centrally. Using the ferret as an animal model for emesis, we performed neuroanatomical and behavioural studies. The presence of the CB1R in the upper gastrointestinal tract was examined by immunohistochemistry combined with anterograde tracing of the vagus nerve. The tracing agent DiI was injected into the nodose ganglion (1µl at 4 sites) in order to visualize the vagal afferent fibers in the stomach and duodenum of the ferret after 4-6 weeks. Immunohistochemistry for CB1R in the stomach and duodenum of these animals showed that CB1R are present on intrinsic nerves of the gut, but not on the vagal afferent fibers. In order to test the site of action behaviourally, a low dose of delta 9 tetrahydrocannabinol (THC) was administered intracisternally in anesthetized ferrets before intragastric gavage with hypertonic saline (10ml over 10min) as an emetic stimulus. The same emetic stimulus was used in a different group of animals to examine the effect of cannabinoids on neuronal activation in the brainstem using Fos immunohistochemistry. Intracisternal administration of a peripherally inactive dose of THC (0.01 mg/kg) blocked the effect of the emetic stimulus. In control experiments with the drug vehicle administered intracisternally, intragastric hypertonic saline caused an average of 4.0 episodes (n=2) of retching with a coincident rise in blood pressure. Pretreatment with THC resulted in no episodes of retching and no rise in blood pressure. Fos immunoreactive cells were counted in the dorsal vagal complex of the brainstem in response to intragastric hypertonic saline. Pretreatment with the CB1R agonist WIN55,212-2 (1 mg/kg, i.p., n=4 ferrets) reduced Fos expression in the dorsal motor nucleus of the vagus 1mm rostral to obex (12.3 ± 1.5 to 3.5 ± 1.0 cells/section). At the level of the obex, Fos expression was reduced in the medial subnucleus of the nucleus of the solitary tract (46.3 ± 5.7 to 12.0 ± 0.9 cells/section) as well as in the area postrema (24.3 ± 1.6 to 4.3 ± 1.4 cells/section). The nucleus of the solitary tract also showed a reduction in Fos expression in the commissural subnucleus 0.5mm caudal to obex (36.0 ± 5.8 to 10.0 ± 3.0 cells/section). These results support the hypothesis that in the ferret, the site of the anti-emetic action of cannabinoids is in brainstem.

ENDOCANNABINOIDS AND COLORECTAL CARCINOMA: A POSSIBLE ROLE IN THE TONIC INHIBITION OF CANCER GROWTH

Vincenzo Di Marzo[1], Tiziana Bisogno[1], Alessia Ligresti[1], Isabel Matias[1], Luciano De Petrocellis[2], Giuseppe D'Argenio[4], Gaetano Iaquinto[5], Maurizio Bifulco[3], and Italo Sorrentini[6]

[1-3]*Endocannabinoid Research Group,* Institutes of [1]Biomolecular Chemistry and [2]Cybernetics, C.N.R., Pozzuoli, (NA), Italy, [3]Dipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno, Fisciano, (SA), [4]Gastroenterologia, Università "Federico II", Napoli, [5]Gastroenterologia e Anatomia Patologica, A.O. "Moscati", Avellino, and [6]Gastroenterologia, A.O. "Rummo", Benevento, Italy

The endocannabinoids, arachidonoyl-ethanolamide (AEA, anandamide) and 2-arachidonoylglycerol (2-AG), have been previously shown to potently inhibit the proliferation of cancer cells in vitro and in vivo by acting at cannabinoid receptors type 1 (CB_1). Here we have investigated the possible role of AEA and 2-AG in colorectal carcinoma by measuring, in tumour tissue and neighbouring normal colon mucosa, the levels of these compounds, of their receptors, CB_1 and CB_2, and of the enzyme mostly responsible for their degradation, the fatty acid amide hydrolase (FAAH). Biopsies on both healthy and cancer tissue were obtained by means of biopsy forceps during colonoscopy in 9 patients affected with left-sided colon carcinoma, and kept at –80°C until processing. Aliquots of each sample were also stored in formalin for histology to evalutate cancer grading, mitoses for high power field and nuclear pleiomorphism. Endocannabinoid levels in lipid extracts were measured by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS). The expression of CB_1, CB_2 and FAAH mRNA was measured by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). CB_1 protein expression was also assessed by Western immunoblotting.

We found that both healthy and tumour colon tissues contain AEA and 2-AG and express CB_1 and CB_2 receptors as well as FAAH. Moreover, the levels of the two endocannabinoids, but not of CB_1, CB_2 and FAAH, were significantly increased in cancer tissue as compared to normal neighbouring mucosa (AEA 1.18$\pm$0.21 vs. 0.55$\pm$0.11 pmol/mg lipid extract, $P<0.005$; 2-AG 0.56+0.11 vs. 0.23+0.06 nmol/mg lipid extract, $P<0.005$, Student's paired T test, means $\pm$ SEM, n=9). These findings suggest that the endocannabinoid system might play a function in the growth of colorectal cancer. Therefore, we examined the effect of AEA on the growth of the human colon carcinoma cell line CaCo-2. We found that these cells also express CB_1 and CB_2 receptors as well as FAAH, and that AEA inhibits dose-dependently the growth of CaCo-2 cells in culture (IC_{50}=0.5 microM). The effect of AEA was blocked by the CB_1 receptor antagonist SR141716A (0.2 microM) and was reproduced by the selective CB_1 agonist, arachidonoyl-chloro-ethanolamide (IC_{50}=0.1 microM). Our data suggest that endocannabinoids are over-produced by colon tumours where they might play a tumour suppressing action by activating CB_1 receptors.

METHANANDAMIDE TREATMENT INCREASES MURINE LUNG CANCER GROWTH IN IMMUNOCOMPETENT MICE BY A CANNABINOID RECEPTOR INDEPENDENT PATHWAY

Brian Gardner, Li X. Zhu, Sherven Sharma, Donald P. Tashkin, and Steven M Dubinett.
Pulmonary Immunology Laboratory, UCLA School of Medicine and VA
West Los Angeles Healthcare Center, Los Angeles, CA

We have previously shown that Δ^9-tetrahydrocannabinol (THC), the major psychoactive component of marijuana, suppresses host immune responses against weakly immunogenic murine lung cancers. Administration of THC (5 mg/kg, four times/wk i.p.) led to accelerated growth of tumor implants compared to treatment with diluent alone. In contrast to our findings in immunocompetent mice, THC did not affect tumor growth in tumor-bearing SCID mice. The immune inhibitory cytokines, IL-10 and TGF-β, were augmented, while IFN-γ was down regulated at both the tumor site and in the spleens of THC-treated mice. In vivo administration of the CB_2 cannabinoid receptor antagonist SR144528 blocked the THC mediated increased tumor growth. In order to investigate the role of the endocannabiniod system in regulation of the immune response to lung cancer we evaluated the effect of methanandamide treatment on tumor growth. We found that methanadamide administration (5 mg/kg, four times/wk i.p.) resulted in an increased rate of tumor growth compared to diluent treated controls. Methanandamide administration resulted in increased production of splenocyte IL-10 and decreased IFN-γ as was observed with THC treatment; however TGF-β levels were not increased. In addition methanandamide treatment increased production of PGE2. In contrast to what we observed with THC treatment, methananamide-mediated augmentation of tumor growth was not blocked by the CB_2 receptor antagonist SR144528. The CB_1 receptor antagonist, SR141716 also did not inhibit methanandamide enhanced tumor growth. Our results suggest that in contrast to THC, methanandamide treatment results in increased tumor growth by a cannabinoid receptor independent pathway.

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CANNABINOID RECEPTOR STIMULATION IN THE SEPTUM OR THE HIPPOCAMPUS DIFFERENTIALLY AFFECTS THE ACTIVITY OF THE SEPTOHIPPOCAMPAL CHOLINERGIC PATHWAY

Eleni T. Tzavara, Mark Wade and George G. Nomikos
Eli Lilly and Company, Lilly Corporate Center, Neuroscience Discovery Research, Indianapolis, IN 46285-0510

Cannabinoids represent a new class of promising therapeutic molecules with indications such as spasticity (multiple sclerosis), stereotypies (Gilles de la Tourette), brain trauma, chronic pain, glaucoma, and appetite and mood stimulation. Their therapeutic potential can however be restricted because of side effects, the most important being memory impairments, reported during recent clinical trials as well as in primate and rodent experimental models. Cannabinoids modulate neurotransmitter release and synaptic plasticity, in a number of brain regions, including the hippocampus, which plays a crucial role in cognition and bears a high density of cannabinoid (CB_1) receptors. In the hippocampal slice, cannabinoids have been shown to decrease LTP and LTD, to mediate depolarization-induced suppression of inhibition, and to inhibit GABA and acetylcholine (ACh) release. The aim of this study was to investigate the functional interactions between cannabinoids and the septohippocampal pathway. In vivo microdialysis experiments were compliant to the guidelines of the Animal Care and Use Committee of Eli Lilly and Co. Wistar rats (250-300g) were implanted with concentric microdialysis probes (BAS, BR-4) in the hippocampus. For some studies, rats were also implanted in the medial septum (BAS, BR-2). About 24 hr later, a modified Ringer's solution containing 0.1 μM neostigmine was perfused at a rate of 2.4 μl/min and samples were collected every 15 minutes. For double implantation studies, a modified Ringer's solution was also perfused in the septum, at a rate of 2.4 μl/min. Dialysates were analyzed for ACh with on-line HPLC/electrochemical detection. Drugs were dissolved in 2%DMSO, 2% cremophor EL, and saline for i.p. injection or in 0.5% DMSO, 0.5% cremophor EL, perfusion solution, for local infusion through the probe. We found that cannabinoids exert a biphasic action on ACh release from the hippocampus; at a low dose (0.5mg/kg i.p.) they have a transient stimulatory effect while at a high dose (5 mg/kg i.p) they have a much more prolonged inhibitory effect, the stimulatory component being markedly attenuated. The inhibitory effects of the high dose are mediated locally in the hippocampus, since intra-hippocampal infusion of the CB_1 receptor antagonist SR141716A (at a concentration of 100μM that has no effects per se) completely abolishes this effect. The stimulatory effects of the low dose are mediated in the medial septum/diagonal band of Broca, where the majority of cholinergic cell bodies projecting to the hippocampus reside, because SR141716A (100 μM) infused in the septum abolishes the stimulatory effects of the low dose, without affecting the inhibitory effects of the high dose. The inhibitory and stimulatory effects of cannabinoids involve different neuroanatomical sites. We also found that intra-septal infusions of the cannabinoid agonist WIN55212-2 (100μM) increased hippocampal ACh, but decreased ACh content in the septum. We, thus, propose that the stimulatory effects of cannabinoids on hippocampal ACh may be due to disinhibition of the cholinergic neurons at the level of the septum via a tonically active autoregulatory loop.

AN ENDOCANNABINOID SYSTEM MODULATES LEARNING AND MEMORY IN MICE AS ASSESSED IN THE MORRIS WATER MAZE

Aron H. Lichtman, Stephen A. Varvel, and Emmanuel Anum
Department of Pharmacology and Toxicology, PO Box 980613,
Richmond, VA 23233-0613, USA

An endocannabinoid system in the brain consisting of CB_1 receptors and endocannabinoids has been proposed to modulate a variety of physiological functions including pain, feeding, drug dependence, and learning and memory. The goal of the present study was to elucidate the role that this system plays on different components of cognition as assessed in the Morris water maze, a well characterized spatial cognitive task. This task involves placing a subject in a water-filled pool containing an escape platform that is submerged 1 cm beneath the surface of the water. Subjects learn the location of the platform and navigate towards it by using distal visual cues. The two main dependent measures of interest are escape latency (i.e., the time to find the platform) and path length (i.e., the total distance traveled). In order to assess whether learning and memory is under endocannabinoid tone, we compared Morris water maze performance between wild type control ($CB^{+/+}$) mice and mice devoid of CB_1 receptors (i.e., $CB_1^{-/-}$ mice). Both groups exhibited identical performance during seven acquisition sessions, indicating no apparent phenotype differences in learning the task. Subsequently, both genotypes were given a reversal session in which the platform was moved to the opposite end of the tank and subjects were given four two-min trials to learn the new location of the platform. Whereas the $CB_1^{+/+}$ group decreased both escape latencies and swim paths over subsequent trials indicating that they learned the new location of the platform, the $CB_1^{-/-}$ group unexpectedly failed to exhibit any such improvement. This apparent learning deficit in the $CB_1^{-/-}$ mice was accounted for by the fact that they continued to return to the previous location of the platform, while the $CB_1^{+/+}$ mice decreased the number of returns. The propensity of the $CB_1^{-/-}$ mice to return to the previous platform location suggests that endocannabinoids serve to facilitate processes related to either forgetting or extinction. In order to test these two possibilities, subjects were repeatedly treated with either vehicle or the CB_1 antagonist SR141716A (3 mg/kg) and evaluated for acquisition across eight sessions, extinction in which the platform was removed for five consecutive sessions, reinstatement that consisted of a single session, and three reacquisition sessions in the Morris water maze. Both vehicle-treated and SR141716A-treated mice exhibited identical acquisition and extinction rates. However, consistent with the notion that antagonizing CB_1 signaling improves memory, the drug-treated mice returned to the location at which the platform had been located more often than the controls during the first extinction trial. In the reinstatement session, the platform was temporarily returned to its original location and each mouse was placed directly on it for 30 seconds, then removed, given its appropriate injection, and evaluated with procedures identical to the previous five extinction sessions. The SR141716A-treated group exhibited improved performance compared to the control group during both the reinstatement session and the reacquisition sessions. Collectively, these findings support the hypothesis that the endocannabinoid system functions to facilitate the forgetting of irrelevant or maladaptive behaviors. Thus our findings suggest that blockade of endocannabinoid signaling enhances memory of well-learned behaviors, but can interfere with learning new responses in tasks that require the suppression of these well-learned responses, as in the reversal task.

EXTINCTION OF AVERSIVE MEMORIES IS CONTROLLED BY THE ENDOGENOUS CANNABINOID SYSTEM

Giovanni Marsicano[1], Carsten T. Wotjak[2], Shahnaz C. Azad[3], Tiziana Bisogno[4], Gerhard Rammes[3], Maria Grazia Cascio[4], Heike Hermann[1], Jianrong Tang[2], Clementine Hofmann[1], Walter Zieglgänsberger[3], Vincenzo Di Marzo[4], and Beat Lutz[1]
Groups [1]Molecular Genetics of Behaviour, [2]Neuronal Plasticity, and [3]Neuropharmacology
Max-Planck-Institute of Psychiatry, 80804, Munich, Germany
[4]Endocannabinoid Research Group, Institute of Biomolecular Chemistry, CNR, 80078 Pozzuoli (Napoli), Italy

The ability to rapidly acquire and store memory of aversive events is one of the basic principles of central nervous systems throughout the animal kingdom. In the absence of reinforcement, the resulting behavioural response of the organism will gradually diminish to be finally extinct. Despite the importance of extinction, its cellular mechanisms are largely unknown. The cannabinoid receptor type 1 (CB_1) and endocannabinoids are present in memory-related brain areas and modulate memory processing. Here we show that the endogenous cannabinoid system plays a central role in the extinction of aversive memories. CB_1-deficient mice showed strongly impaired short-term and long-term extinction in an auditory fear-conditioning test, with unaffected memory acquisition and consolidation. Treatment of wild-type mice with the CB_1-specific antagonist SR141716A mimicked the phenotype of the CB_1-deficient mice, and revealed that CB_1 is required at onset of memory extinction. Consistently, tone presentation during extinction trials resulted in elevated levels of endocannabinoids in the basolateral amygdala complex, one of the brain areas controlling extinction of aversive memories. In the basolateral amygdala, endocannabinoids and CB_1 were crucially involved in long-term depression of GABAergic inhibitory currents. We propose that endocannabinoids facilitate extinction of aversive memories via their selective inhibitory effects on local GABAergic networks in the amygdala.

ACTIVATION OF CANNABINOID RECEPTORS IN THE PERIAQUEDUCTAL GREY MODULATES BEHAVIOUR IN A RAT MODEL OF AVERSION

David Finn, Maulik Jhaveri, Simon Beckett, David Kendall,
Charles Marsden, and Victoria Chapman
Neuroscience and Pharmacology Group, School of Biomedical Sciences,
University of Nottingham Medical School, Queen's Medical Centre,
Nottingham, NG7 2UH, United Kingdom

Despite undoubted therapeutic potential, cannabis and cannabinoid drugs induce aversive responses including anxiety and panic attacks in some individuals. The midbrain periaqueductal grey (PAG) mediates behavioural responses to aversive stimuli (Bandler *et al*., **2000**. *Brain Res. Bull.*, **53,** 95-104). The PAG contains a moderate density of CB_1 receptors which may mediate some of the aversive effects observed following cannabis consumption. Stimulation of the rat PAG with the excitatory amino acid D,L-homocysteic acid (DLH) induces aversive behaviour characterised by explosive running and jumping or quiescence (Beckett *et al*., **1992**. *Psychopharmacology*, 108, 110-114). We have investigated the effects of intra-PAG administration of the CB agonist HU210 on DLH-induced aversive behaviour. Male Sprague-Dawley rats (250-300 g) were cannulated in the dorsal PAG under isoflurane/N_2O anaesthesia. Rats received an intra-PAG microinjection of HU210 (0.1, 1 or 5 μg/250 nl) or vehicle (250 nl 60% DMSO in aCSF) 6-7 days post-surgery. Behaviour was tracked for 10 min post-HU210/vehicle and for 5 min after an intra-PAG injection of DLH (5nmol in aCSF). Post-DLH data were split into two 2.5 min time-bins. Distance moved, velocity, grooming, rearing, jumping and freezing behaviour were measured. Data were analysed by one-way ANOVA followed by Fisher's PLSD post hoc test. Two distinct types of behaviour were observed following DLH administration. 71% of rats exhibited explosive running and jumping behaviour (group A) while 29% exhibited quiescence (group B). In group A rats, HU210 (5 μg/250 nl) significantly reduced the maximum velocity during the first 2.5 min post-DLH compared with vehicle controls (Table 1a). HU210 had no significant effect on the distance moved during the first 2.5 min post-DLH (Table 1a). The distance moved 2.5-5 min post-DLH was significantly less in HU210 (0.1, 1 and 5 μg/250 nl) treated animals compared with vehicle controls as was the maximum velocity of movement (Table 1b). In contrast, the locomotor activity of rats exhibiting quiescence post-DLH was not significantly affected by HU210 pre-treatment. HU210 did not significantly affect grooming, rearing, jumping or freezing behaviour post-DLH in either group A or B rats compared with controls.

(a)	Vehicle	HU210 (0.1)	HU210 (1)	HU210 (5)
Distance (cm)	2448 ± 223	3512 ± 699	2895 ± 820	1868 ± 767
Max Velocity (cm/s)	169 ± 10	168 ± 9	136 ± 17	122 ± 18*
(b)				
Distance (cm)	934 ± 243	220 ± 131*	217 ± 100*	203 ± 59*
Max Velocity (cm/s)	39 ± 7	11 ± 4**	11 ± 4.7**	17 ± 10*

Table 1. Effect of intra-PAG HU210 (0.1, 1 and 5 μg/250 nl) on locomotor activity of rats exhibiting explosive behaviour (a) 0-2.5 min and (b) 2.5-5 min post-DLH. Values are mean ± s.e. mean (n = 6-14). $^{*}P < 0.05$, $^{**}P < 0.01$ vs vehicle treated controls.

Our data demonstrate that functional CB receptors located in the PAG can modulate a specific set of DLH-evoked aversive responses. The inhibitory effects of HU210 on the explosive behaviour may be due to cannabinoid mediated inhibitory modulation of efferent pathways involved in the aversive behavioural response.

DETERMINATION OF CB_1 RECEPTOR ANTAGONIST ANORECTIC EFFICACY VS ADIPOSE TISSUE MASS REDUCTION

Shawn C. Black, Audrey L. Hildebrandt, and Dawn M. Kelly-Sullivan
Department of Cardiovascular and Metabolic Diseases,
Pfizer Global Research and Development, Eastern Point Road Groton,
Connecticut, 06340, USA

Acute anorectic effects of cannabinoid receptor 1 (CB_1) antagonists in rodents are well established and this class of compounds are proposed as potential anorectic drugs to treat human obesity. Studies have demonstrated that cannabinoid antagonists are anorectic and reduce body weight in lean, growing rats over a two-week period (Columbo et al Life Sci 63: 113-117, **1998**). However the effect of a CB_1 receptor antagonist in an obese rodent model of stable, obese body weight has not been reported. Thus we sought to determine the anorectic and weight loss efficacy of CB_1 receptor antagonists in a diet-induced obese (DIO) mouse model. Furthermore, we determined the effect of CB_1 receptor antagonist treatment on in situ adipose tissue mass, using a novel methodology, microCT. Male C57/Bl6 mice were placed on a high fat diet (45% of kcal derived from fat, Research Diets D12451) for a period of 11 weeks, to produce a stable DIO state. Following a one-week oral dosing acclimation period, mice (9-10/group) were treated with vehicle (0.5% methylcellulose) or 30 mg/kg/day of AM251. The effect of CB_1 receptor antagonist treatment on food intake and body weight was determined daily. Mice were dosed once/day 1 hr prior to the dark cycle. Micro CT was used to measure in situ adipose tissue mass at baseline and after 7 days CB_1 receptor antagonist treatment. There were significant reductions in food intake and body weight after a single dose of AM251. There was a time-dependent reduction in the magnitude of the anorectic effect of the CB_1 receptor antagonist: anorectic efficacy was attenuated on day 4 with AM251 at 30 mg/kg/day. In spite of the attenuation in the anorectic efficacy of the CB_1 receptor antagonist, the reduction in body weight observed with AM251 remained significantly less than vehicle-treated mice throughout the one-week study period. Dosing of AM251 at 30 mg/kg/day lead to a significant reduction in the mass of the inguinal subcutaneous, mesenteric, retroperitoneal and epididymal adipose tissue depots. These studies confirm the anorectic efficacy of a CB_1 receptor antagonist and additionally demonstrate that weight loss is associated with a reduction in inguinal subcutaneous, mesenteric, retroperitoneal and epididymal adipose tissue masses.

CORTICOTROPIN-RELEASING HORMONE MEDIATES THE INHIBITORY ACTIONS OF CANNABINOIDS ON FEEDING BEHAVIOR IN FEMALE RATS

Laura L. Murphy, Adam Campbell, Christopher Reis,
Mandy Walters, Kimberly Branson, and Timothy Reis
Department of Physiology, Southern Illinois University
School of Medicine, Carbondale, IL 62901, USA

It is well documented that acute cannabinoid administration stimulates the hypothalamo-pituitary-adrenal hormone axis. Increases in corticotropin-releasing hormone (CRH) can produce hormonal, metabolic and behavioral changes in experimental animals. Notable, is the anorexigenic effect of CRH observed following sustained stress or after intracranial administration of CRH. Whereas low doses of cannabinoid have been shown to stimulate eating behavior, doses of cannabinoid that significantly increase stress hormone levels also inhibit feeding behavior. The present study was designed to examine if CRH is responsible for the inhibitory effects of acute cannabinoid administration on feeding behavior.

Adult female Sprague-Dawley rats were ovariectomized and, four weeks later, implanted with polyethylene cannulae into the right jugular vein. Approximately 24 hr post-cannulae insertion, rats (≥10 animals/treatment group) were treated iv with HU-210 (0.5, 2.0 or 20 µg/kg; Tocris), CP55,940 (6.25, 12.5 or 25 µg/kg; Tocris) or vehicle (5% Tween-80, 5% propylene glycol in 0.9% saline). Immediately prior to treatment (10:00h) and 60 min post-treatment blood was obtained via jugular cannulae for hormone measurements. For monitoring feeding behavior, rat chow and water were restricted for 1 hr prior to treatment and returned to cage immediately after treatment. The following parameters were obtained during 60 min observations: change in body weight, number of feeding and drinking episodes, and amount of food and water consumed. In results, the lowest doses of cannabinoid produced significant increases in body weight ($p<0.05$) and number of feeding ($p<0.01$) and drinking episodes ($p<0.05$) relative to vehicle controls. The highest cannabinoid doses significantly decreased body weight ($p<0.001$) and amount of food and water consumed ($p<0.05$) compared to vehicle controls. The intermediate doses of cannabinoid had no effects relative to controls. There was a dose-related increase in plasma ACTH and corticosterone levels 60 min after cannabinoid administration.

In a second study, icv cannulae were inserted into the third ventricle of anesthetized rats one week prior to testing. The CRH antagonist D-phe-CRF (0.2µg/5µl; Sigma) or saline was infused 10 min prior to iv CP55,940 (25µg/kg) or vehicle and feeding behavior was monitored. Results showed that pretreatment with D-phe-CRF prevented the inhibitory effects of high dose cannabinoid on change in body weight ($p<0.05$), no. of eating episodes (veh, 3.9±0.5; D-phe, 13.3±2.1; $p<0.001$), and amount of food consumed (veh, 1.6±0.3; D-phe, 3.9±0.5gm; $p<0.01$). In fact, in D-phe-CRF treated animals, the increased number of eating episodes and amount of food ingested were not different when compared to low dose cannabinoid-treated animals. Thus, in the absence of CRH, cannabinoids appear to have an overall orexigenic effect. In conclusion, these results indicate that CRH activation by acute high dose cannabinoid administration produces an anorexigenic effect that can be prevented by pre-treatment with D-phe-CRF.

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ROLE OF CB_1 AND "CB_3" RECEPTORS IN NEWBORN FOOD INTAKE AND SURVIVAL

Ester Fride[1], Elena Rosenberg[2], and Raphael Mechoulam[2]
[1]Department of Behavioral Sciences, College of Judea and Samaria, Ariel 44837
[2]Department of Medicinal Chemistry and Natural Products,
Hebrew University Medical Faculty, Jerusalem, Israel 91120.

We have recently suggested that cannabinoid-induced increase in food intake in the mature organism may only be the "tip of the iceberg" of a critical role played by the endocannabinoid system in maternal milk ingestion and hence, survival of the newborn. Thus we have previously shown that blockade of the cannabinoid (CB_1) receptor in one-day old mice pups prevents milk intake and results in death within the first few days of life. Co-administration of tetrahydrocannabinol (THC) reversed the effects of the CB_1 antagonist (SR1417116A).[1] In the present series of experiments, we further investigated whether CB_1 receptors are solely responsible for the devastating effects of SR141716A in mouse pups. To this aim we performed a series of experiments in CB_1 receptor knockout ($CB^{-/-}$) mice[2] and compared the results to those from C57BL6/J wild types.

Methods: In each experiment, weight gain and appearance of milkbands and developmental landmarks including earflap detachment, were recorded over the first 2 weeks of life. Data were calculated on a *per* litter base. In the first experiment, wild type mice were injected with the CB_1 receptor antagonist SR141716A, within 24 h after birth, as previously done in Sabra mice. In the second experiment, the same parameters were recorded for $CB^{-/-}$ and wild type pups, but without pharmacological intervention. In the third experiment, $CB^{-/-}$ pups were treated with the CB_1 antagonist on day one of life and the outcomes were compared to those obtained from SR141716A-treated wild type pups.

Results: **1.** C57BL6/J pups were affected by SR141716A injections at least as potently as Sabra mice. All pups had died by day 4. **2.** Weight gain and pup survival of $CB^{-/-}$ pups were significantly lower compared to wild types, although litter sizes were similar; milkbands were virtually absent on day 1 of life. However **3.** SR141716A only partially affected $CB^{-/-}$ pup growth and survival. Thus mortality was delayed, weight gain was unaffected, while milkband appearance and ear flap detachment were not significantly affected by the CB_1 antagonist.

We conclude from these findings that SR141716A-induced inhibition of neonatal food intake and growth, is mostly, but not completely, a result of specific CB_1 receptor blockade. The partial effect of SR141716A in $CB^{-/-}$ pups supports evidence,[3] that SR141716A inhibits anandamide- or WIN55212-2-induced receptor activation in adult $CB^{-/-}$ mice, thus supporting the existence of an additional ("CB_3") receptor.[4]

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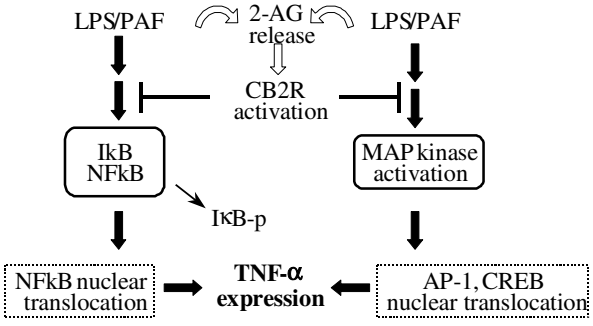
GENERATION AND SIGNALING FUNCTION OF 2-ARACHIDONOYLGLYCEROL IN MACROPHAGES

Evgueni V. Berdyshev, Patricia C. Schmid, Randy J. Krebsbach, and Harald H.O. Schmid
The Hormel Institute, University of Minnesota, Austin, MN55912

We have observed that treatment of mouse P388D1 macrophages with the proinflammatory agonist 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (platelet activating factor, PAF) results in the rapid production and release of 2-arachidonoylglycerol (2-AG). This effect was selective for 2-AG and appeared to be related to phosphatidylinositol (PI) turnover. The level of anandamide (N-arachidonoylethanolamine) remained unchanged during PAF treatment (Berdyshev et al., FASEB J. 15, 2171-2178, 2001). Because activation of cannabinoid receptors is known to cause downregulation of various cell functions, we proposed that PAF-induced 2-AG production plays a direct role in the control of immune cell proinflammatory response. We have now measured the effect of exogenous 2-AG on the PAF- or lipopolysaccharide (LPS)-induced production of tumor necrosis factor-alpha (TNF-α) and PAF-induced ERK activation in P388D1 macrophages. In each case, 2-AG was found to be inhibitory, confirming its proposed role in the control of the proinflammatory response. It is interesting to note that enhanced 2-AG production and release was specific for PAF. Other G-protein-linked or tyrosine-kinase-linked receptor agonists such as sphingosine-1-phosphate, norepinephrine or TNF-α did not affect either 2-AG or anandamide levels, whereas LPS elicited only a very minor response with respect to 2-AG synthesis.

PAF is an important element in the initiation of immune response. Cells are able to synthesize and release it when triggered with different stimuli including LPS and TNF-α. In addition, PAF was shown to prime cells for subsequent stimulation by these factors and is linked through positive feedback relationships with cytokines and eicosanoids. Our findings demonstrate for the first time an important link between the elements of the pro-inflammatory network and 2-AG which may negatively control inflammatory response in an autocrine fashion through the activation of cannabinoid receptors. Identifying the exact mechanisms of this feedback inhibition is important for understanding of the physiological role of the endocannabinoid signaling system.



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THE CANNABINOID CB_2 RECEPTOR PLAYS A ROLE IN IMMUNE FUNCTION

Nancy E. Buckley, Tanya M. Ferguson, and Diep N. Burbridge
Department of Biological Sciences, California State
Polytechnic University-Pomona, Pomona, CA, 91768 USA

The peripheral cannabinoid (CB_2) receptor is predominantly expressed in immune cells. Thus, it is thought that cannabinoid-induced immunomodulation is mediated by this receptor. Using cannabinoid CB_2 receptor knockout mice, we have set out to investigate the role of the cannabinoid CB_2 receptor in immune functions. The immune system is divided into non-specific immune responses, which involve immune cells such as macrophages, and the specific immune response, which involves B and T lymphocytes. It has been reported that cannabinoids alter cytokine expression of macrophages and of T lymphocytes, suggesting that cannabinoids affect both, innate and acquired immunity. It has been reported that interleukin 1βIL1β, IL6 and tumor necrosis factor α (TNFα are altered by cannabinoid treatment of macrophages. Cannabinoids are also known to inhibit Th_1 cytokines interleukin-2 (IL-2) and interferon γ (INFγ) and to enhance Th_2 specific cytokines IL-4 and IL-10. Thus, cannabinoids inhibit the activity of Th_1 cells (cells involved in specific cell mediated immunity), and enhance the activity of Th_2 cells (cells involved in specific humoral activity). To determine whether these effects of cannabinoids on immune cells were mediated by cannabinoid CB_2 receptor, we worked with macrophages and lymphocytes derived from wild type (wt) and cannabinoid CB_2 receptor knockout (ko) mice. Thioglycollate-induced macrophages from wt and ko mice were cultured in vitro and assayed for cytokine production by enzyme-linked immunoassay (ELISA). Splenocytes were also obtained from wt and ko mice and were cultured, assayed for proliferation activity and for specific cytokine production by ELISA. Our results show that the secretion of IL1β, TNFα and IL6 is markedly reduced in macrophages obtained from ko mice as compared to those obtained from wt mice. In the presence of cannabinoids, IL1β, and TNFα levels are differentially altered in macrophages from wt and ko mice. Preliminary work on cytokine profiles of splenocytes derived from wt and ko mice show no difference in IL-4 and IL-10 secretion between wt and ko splenocytes in the presence or absence of cannabinoid. However, INFγ levels in wt and ko splenocytes are differentially affected by cannabinoids. There was no difference in proliferative response to Con A stimulated wt and ko splenocytes. Our findings suggest that the cannabinoid CB_2 receptor plays a role in IL1β, TNFα and IL6 secretion from macrophages and in INFγ secretion from T lymphocytes. Modulation by cannabinoids of the other cytokines may be mediated by the central cannabinoid receptor (in lymphocytes) or by a yet unknown third cannabinoid receptor (in lymphocytes and macrophages). Interestingly, macrophages derived from ko mice have lower levels of anandamide compared to wt macrophages, suggesting that the cannabinoid CB_2 receptor modulates its own endocannabinoids system. We are now investigating the levels of endocannabinoids in ko splenocytes.

EFFECTS OF CANNABIDIOL, A NON PSYCHOACTIVE CONSTITUENT OF MARIJUANA, ON IMMUNE SYSTEM

Paola Massi[1], Angelo Vaccani, Paola Sacerdote[1], and Daniela Parolaro
[1]Department of Pharmacology, Chemotherapy and Toxicology, University of Milan, Via Vanvitelli 32, Milan, Italy and Department of Structural and Functional Biology, University of Insubria, Pharmacology Section, Via A. da Giussano 10, Busto Arsizio, Varese, Italy

Recently there has been renewed interest in developing cannabinoid selective drugs, which might have utility as immunosuppressant or in the treatment of diseases such as arthritis or multiple sclerosis, which are though to be due to inappropriate immune system responses.

Although a vast literature document the in vitro and in vivo immune modulating effects of cannabinoids, their application is however limited by the psychotropic effects that they exert. Cannabidiol (CBD) is one of the major components of marijuana and, unlike the other major components of cannabis, it is devoid of psychotropic effects. Thus CBD appears to be, among the bioactive constituents of Cannabis sativa, one of those with the highest potential for therapeutic use. Although some in vitro reports have shown that CBD can display immunomodulatory properties, little is known about the in vivo effects of CBD on immune cells. Therefore, we undertook the present study evaluating the in vivo effects of CBD on immune system through the functional tests as splenocyte proliferation induced by a mitogen and natural killer cytolytic activity towards YAC-1 tumor cells. It was found that CBD exerted a dose–dependent inhibitory action on the two functional parameters. The dose dependency showed a bell-shaped curve, with the 15 mg/kg i.p. dose exerting the inhibitory effect, whereas both the lowest dose (7.5 mg/kg) and the highest dose (30 mg/kg) were inactive. CBD also interfered with the important function of macrophages such as the ability to migrate towards chemoattractant chemotactic response. Moreover we found that in vivo CBD inhibited in various extension the cells' capacity to produce a wide panel of cytokines representative of a Th1, Th2 and macrophage population responses.

In order to elucidate the mechanism of in vivo CBD action on immune cells, we also performed experiments with the CB_1 and CB_2 receptor antagonists SR141716A and SR144528, respectively. Although CBD, as reported, possess very weak affinity for either cannabinoid receptor subtypes CB_1 and CB_2, both antagonists significantly reversed the CBD-induced inhibition of the immune parameters.

The present results suggest that CBD may be valuable in the treatment of disorders of the immune system and it can represent a new class of agents that could interfere with immune response.

THE NON-PSYCHOACTIVE CANNABIS CONSTITUENT CANNABIDIOL AS ANTI-INFLAMMATORY DRUG IN ANIMAL MODEL IN ACUTE INFLAMMATION

Barbara Costa[1], Silvia Conti[1], Gabriella Giagnoni[1], and Mariapia Colleoni[2]
[1]Dipartimento di Biotecnologie e Bioscienze, Università di Milano-Bicocca, 20126 Milano, Italy; [2]Dipartimento di Farmacologia Chemioterapia e Tossicologia Medica, Università di Milano, 20129 Milano, Italy

Cannabidiol is a non-psychoactive Cannabis constituent and does not bind to either cannabinoid receptor subtype (CB_1 Ki value=4350 nM and CB_2 Ki value=2860 nM). Recent evidences in vitro and in animal models has lead to increasing demonstration that cannabinoids can be employed in the treatment of inflammatory diseases. Particularly, cannabidiol is effective as an anti-arthritic therapeutic, it is able to delay and attenuate symptoms of multiple sclerosis in animal models of these diseases and finally it induces elevated plasma cortisol levels when administered to healthy human volunteers. A recent study reports that cannabidiol is a potent antioxidant that protects rat cortical neurons from glutamate- and reactive oxygen species- induced toxicity without cannabinoid receptor activation. The aim of the present study has been to evaluate whether cannabidiol is able to reduce paw edema and hyperalgesia in a rat model of acute inflammation induced by carrageenan, when orally administered both before and after the induction of inflammation, at different doses (10, 20, 40 mg/kg). Cannabidiol inhibited edema and thermal hyperalgesia in a dose-and time- dependent manner when given 1 h before carrageenan. This cannabinoid is also effective when administered at initiated inflammation once a day for 3 days.

The development of carrageenan inflammation depends on various mediators including prostaglandins , nitric oxide (NO) and free radicals, so we evaluated the influence of cannabidiol treatment on these inflammatory indices. When cannabidiol was given before phlogogen, rats were sacrificed at the inflammation peak (3 h post carrageenan), whereas they were sacrificed 4 days after carrageenan (time at which cannabidiol is more effective) when cannabidiol was therapeutically tested. In both experimental conditions inflamed animals, which received the vehicle of cannabidiol, had high plasmatic levels of prostaglandins E2 (PGE2), and showed in the paw an increase in cyclooxygenase (COX) activity, NO and free radicals content. Thus, we investigated whether the anti-inflammatory effect of cannabidiol was associated with a modification of these classical mediators of inflammation. In animals treated with cannabidiol all these biochemical parameters were significantly lower than those observed in inflamed animals treated with vehicle, and this reduction was dose-related.

These findings suggest that the well known anti-inflammatory and anti-hyperalgesic effect of marijuana could be, at least partially, ascribed to its high cannabidiol content.

Finally, studies are in progress to examine whether cannabidiol anti-inflammatory activity is receptor mediated and/or it depends on its anti-oxidant properties.

AT THE LEADING EDGE: NON-PSYCHOTROPIC CANNABINOID RECEPTORS REGULATE MICROGLIAL MIGRATION

Lisa Walter[1], Allyn Franklin[1], Anke Witting[1], Ken Mackie[2],
George Kunos[3], and Nephi Stella[1,4]
[1]Departments of Pharmacology, [2]Anesthesiology, and [4]Psychiatry,
University of Washington, Seattle, WA, 98195, USA
[3]National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health,
Bethesda, Maryland, 20892, USA

Marijuana contains Δ^9-tetrahydrocannabinol (THC), which produces psychoactive effects through CB_1 receptors, and cannabinol (CBN) and cannabidiol (CBD), which reduce peripheral inflammation through CB_2- and CBD-sensitive receptors, respectively. Whether these three cannabinoids reduce inflammation within the CNS remains controversial – an important issue when considering that multiple sclerosis (MS) patients report benefit from smoking marijuana. Inflammation within the CNS is associated with activation of microglial cells, the resident macrophages of the brain, which migrate towards lesions and exacerbate neuroinflammation. Thus, ingredients that prevent microglial cell migration constitute anti-inflammatory therapeutics.

Here we sought to determine whether THC, CBN, CBD, and endocannabinoids regulate microglial cell migration. Initially, we immunostained primary mouse microglia for the CB_2 cannabinoid receptor. In the presence of treatment with cannabinoid agonists and antagonists, microglial migration was measured using the Boyden chamber, and MAP-kinase activity was quantified by Western blot. Finally, microglial and neuronal endocannabinoid production in response to pathophysiological stimuli was measured by gas chromatography/mass spectrometry (GC/MS).

CB_2 cannabinoid receptors were highly expressed at the leading edges of microglial cell lamellipodia. Agonist activity at CB_2 and CBD-sensitive receptors modulated microglial migration and activated the MAP-kinase cascade. Both microglia and neurons produced two well-known endocannabinoids, anandamide and 2-arachidonylglycerol, as well as two other congeners of anandamide, docosatetraenylethanolamide and homo-γ-linolenylethanolamide, all of which regulated microglial migration by acting through CB_2- and CBD-sensitive receptors. Thus, our study identifies a cannabioid signaling system in microglia that regulates their migration and suggests a non-psychotropic, cannabinoid-based, anti-inflammatory target in the central nervous system.

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IN SEARCH OF PLANTS, OTHER THAN *CANNABIS SATIVA*, WITH CANNABINOID RECEPTOR ACTIVITY

E. Russo[1], S. Ben-Shabat[2,3], E. Fride[4], K. Parker[5], and R. Mechoulam[2]
[1]Montana Neurobehavioral Specialists, 900 North Orange, Missoula, MT 59802, USA
[2]Department of Natural Products, Medical Faculty, Hebrew University, Jerusalem, Israel
[3]Institute for Applied Research, Ben-Gurion Univ. of the Negev, Beersheva 84105, Israel
[4]Department of Behavioral Sciences, College of Judea and Samaria, Ariel 44837, Israel
[5]Department of Pharmaceutical Sciences, University of Montana,
Missoula, MT 59812, USA

Salvia divinorum is a perennial plant of the Lamiaceae Family, native to the Mazatecan Sierra of Mexico, where it is employed by indigenous peoples as an agent of divination and as an herbal treatment for headaches and other ills. The primary active ingredient has been identified as salvinorin A, a potent psychoactive diterpenoid agent devoid of nitrogen, analogous in some respects to THC. Examination of salvinorin A by NovaScreen® revealed no affinity for most known neurotransmitter receptors (Siebert, J. Ethnopharmacol., **1994**, 43, 53-6). The current study examined possible cannabinoid (CB_1) activity of *Salvia divinorum* leaves and salvinorin A. The leaves were purchased from two commercial sources and 98% pure salvinorin A from D. Siebert . The leaves (700 mg) were extracted first in petroleum ether (PE) to give the non-polar extract. The residue was re-extracted in ethyl acetate and afterward with methanol to give the polar extracts. The extracts were evaluated for CB_1 receptor binding. The samples for the binding assay were carried out in a gradient ranging from 1/10 up to 1/1000 of the total extract. The PE extract was found to be active at the CB_1 receptor while the other two extracts (EtOAc and MeOH) were not. The PE extract was purified by open column chromatography and by glass-backed TLC to give a relatively pure fraction where by GC-MS showed an unidentified peak with a mass of m/z 340. The *Salvia divinorum* extraction was carried out twice in two independent experiments (700 mg in each experiment) and the binding results were the same. Pure salvinorin A was inactive in the CB_1 receptor assay in concentrations up to 10μM. We have not been able to detect THC or its derivatives in any of extracts. We also examined *Columnea ericae* Mansfeld, a neotropical herb of the Gesneriaceae Family, native to Amazonian regions of South America. Its use by indigenous peoples as an abortifacient, and treatment for menorrhea and snakebite, suggest possible anti-inflammatory or ergot-like properties. Its biochemical activity is virtually unexplored; anecdotal evidence suggests a short-lived psychoactive effect akin to that of cannabis, with a mild feeling of calm and increased sensory acuity when smoked. A vouchered specimen of *Columnea ericae* (94-022) was procured from the Smithsonian Institution and cultivated. We employed a 5HT2A receptor assay using transfected NIH 3T3 cells (a gift of Dr. D. Julius, UCSF). Receptors were assayed from crude membrane preparations in the presence of 0.2 nM [3H]ketanserin. Non-specific binding was determined in the presence of 10 μM mianserin. Crude plant extracts were assayed at 1/200 final dilution and revealed approximately a 40% displacement of ketanserin. No cannabinoid (CB_1) binding activity of the crude extract (assayed as for *Salvia*) was detected. We conclude that *Salvia* may have cannabinoid-like activity, while *Columnea* is more likely to have serotonin-like activity.

CANNABIS AND CANNABINOID RECEPTORS: THE CHICKEN-OR-EGG QUESTION

John M. McPartland, and Geoffrey Guy
GW Pharmaceuticals, Porton Down Science Park, Salisbury, Wiltshire, SP4 0JQ, UK

Because angiosperm plants evolved early in the Cretaceous period (100 millions years ago) and modern humans emerged a mere 100,000 years ago, pundits have suggested, "humans surreptitiously evolved CB receptors that bind to *Cannabis* compounds." We addressed this adage by examining the phylogenetic history of CB receptors, coupled with a novel approach for dating the epoch of *Cannabis* evolution.

Binding studies with [^{3}H]CP55,940 and other CB ligands show that CB receptors are present in many animals besides humans, including other mammals, birds, amphibians, fish, sea urchins, molluscs, leeches, and even *Hydra vulgaris*. The genes from some of these animals have been cloned and sequenced. These sequences were used to construct a phylogenetic CB gene tree, which serves as a stochastic "molecular clock" for the timing of evolutionary events. The gene tree is rooted by an ancestral CB gene that underwent a duplication event, giving rise to present-day CB_1 and CB_2. The sequence divergence between CB_1 and CB_2 (47% identity) is greater than that between human CB_1 and the partially-sequenced leech CB_1 (58% identity), suggesting the duplication event predated the divergence of vertebrates and invertebrates. This evidence suggests the primordial CB receptor evolved at least 600 million years ago. We hypothesize that the primordial CB receptor diverged from a related G-protein coupled receptor, and it linked with a pre-existing ligand, anandamide. Anandamide acts as an agonist at CB receptors as well as vanilloid (VR) receptors. Our phylogenetic analysis suggests that VR receptors evolved before CB receptors, so anandamide first served as a VR ligand.

Because fossil records of *Cannabis* are lacking, the epoch when it evolved must be inferred from fossils of related plants. Unfortunately, kinship relationships remain unresolved; taxonomists shuttle *Cannabis* between the *Moraceae* and the *Urticaceae.* We examined this issue by utilizing parasites as "nonhuman taxonomists." Many obligate parasites coevolve with their hosts, eventually becoming dependent on a restricted number of related species. Thus, parasite phylogeny mirrors host phylogeny. We compiled a monograph of *Cannabis* parasites (fungi, nematodes, insects) and compared it with lists of parasites that attack other plants. Seven *Cannabis* parasites were shared by hosts in the *Urticaceae,* whereas no obligate parasites of *Cannabis* were shared by *Moraceae.* hosts. These results suggest that *Cannabis* has closer ties with the *Urticaceae* (with fossils dating to the Oligocene epoch, 34 million years ago), than with the *Moraceae* (fossils dating to the early Eocene, 56 million years ago). Thus *Cannabis* is a relatively young species, appearing about the same time as the kiwi.

In conclusion, CB receptors evolved long before than *Cannabis*. The aforementioned aphorism is incorrect. Indeed, the reverse is true – "*Cannabis* surreptitiously evolved compounds that bind to CB receptors."

NATIONAL INSTITUTE ON DRUG ABUSE
DRUG SUPPLY AND ANALYTICAL SERVICES PROGRAM

Hari H. Singh, and Rao S. Rapaka
Chemistry and Physiological Systems Research Branch, Division of Neuroscience
and Behavioral Research, National Institute on Drug Abuse, NIH,
Bethesda, MD 20892-9555 USA

The NIDA Drug Supply Program has grown from a source of marijuana, LSD, and a small number of research compounds in the early 1970's to the major source of research compounds for the drug abuse research community. Initially, the Drug Supply Program focused on THC and other naturally occurring cannabinoids, and then gradually began to fulfill the demand for metabolites of these compounds, various isotope-labeled derivatives, and other drugs of abuse. The Drug Supply Program has been responsive to the needs of the research community over the past years when a variety of new compounds were required: opiate antagonists, phencyclidine derivatives, cocaine metabolites and analogs, and a variety of receptor ligands for studies of opiate receptors. When "designer drugs" became of interest to researchers, meperidine and fentanyl derivatives, and more recently, new substituted amphetamines were synthesized in response to the interest generated by the increased abuse of MDMA on college campuses.

Since starting in 1967 with only LSD, hundreds of different compounds were made available to the research community. Today, the Drug Supply Program provides basic research investigators with authentic stimulants, hallucinogens, narcotics, cannabinoids, opioid peptides, and marijuana. Labeled compounds are also available which incorporate deuterium, tritium, 14C or 125I isotopes. New compounds are periodically added to the Drug Supply inventory as needed by the research community. Resources updates which list newly added or deleted chemicals and/or services are mailed to investigators upon request.

In addition to the Drug Supply Program, research resources are provided for the quantitative analysis of drugs of abuse by RIA or GC/MS, and determination of 3-dimensional structure parameters by x-ray crystallography. The Drug Supply and Analytical Services Program also provides analytical support for the analysis of cannabinoids by providing quantitative stock solutions to investigators.

Personnel at the NIDA Drug Supply and Analytical Services Program at the Division of Neuroscience and Behavioral Research are available to answer your questions and to assist you in using these services. In order to utilize the services or obtain materials offered by NIDA, interested research investigators are required to submit their requests along with research protocols detailing the work to be carried out. The protocols are reviewed by NIDA staff and/or external experts for scientific merit and compatibility with NIDA's goals. If the research protocols meet basic requirements, the requests are processed subject to resource availability.

CANNABINOID AGONISTS AND ANTAGONISTS MODULATE CONDITIONED GAPING: A RAT MODEL OF NAUSEA

Linda A. Parker[1], and Raphael Mechoulam[2]
[1]Department of Psychology, Wilfrid Laurier University, Waterloo, Ontario, Canada.
[2]Department of Medicinal Chemistry and Natural Products,
The Hebrew University of Jerusalem, Israel.

Considerable evidence indicates that conditioned gaping in rats reflects nausea in this species that does not vomit. We have reported that the non-psychoactive cannabinoids, cannabidiol and its dimethylheptyl homolog, interfere with the establishment and the expression of lithium-induced conditioned gaping in rats. Here we present evidence that the cannabinoid agonists THC and HU-210 also interfere with the establishment of lithium-induced conditioned gaping in rats. This interference is reversed by pretreatment with the CB_1 antagonist, SR141716A. Finally, SR141716A potentiates gaping when administered prior to conditioning with lithium, suggesting that endogenous cannabinoids modulate nausea in rats.
tested for antidepressant effects.

CANNABINOID TREATMENT AND CB_1 RECEPTOR INVOLVEMENT IN INFLAMMATORY DISEASE IN THE CNS

Gareth Pryce, Deborah Hankey, Zubair Ahmed, and David Baker.
Department of Neuroinflammation, Institute of Neurology,
University College, London, WC1N J, United Kingdom

There is increasing interest in the use of medical cannabis in multiple sclerosis (MS). Whilst many people claim symptomatic relief, it has been suggested that there could also be beneficial effects on disease progression. Using an experimental autoimmune encephalomyelitis (EAE) mouse MS model we have been able to demonstrate tonic cannabinoid-mediated control of spasticity, which occurs during chronic disease (Baker et al **2000**. *Nature* 404:84). We have used this system also to investigate whether cannabinoids can also alter the paralytic disease progression. During active clinical disease, excessive gluatamate release has been associated with exocitoxicity of neurons leading to cell death (Smith et al **2000**. *Nat. Med* 6:62). The cannabinoid system functions as a regulator of neurotransmitter, including glutamate, release (Wilson & Nicoll, *Nature* **2001**, 410:588). This indicates that the cannabinoid system may offer some neuroprotective function, which could limit accumulation of neurological deficit. One route is to apply exogenous cannabinoid agonists, antagonists and monitor influences on disease, whilst another approach is to examine the influence of disease in CB receptor gene-deficient mice. Autoimmune disease of the brain and spinal cord (EAE) or eye (autoimmune uveitis (EAU)) was induced following active immunization of myelin/retinal antigens in Freund's adjuvant. Clinical paralysis was visually assessed (Baker et al **1990**. *J.Neuroimmunol* 28**:**261) and retinal infiltration was assessed histologically in wax sections (Hankey et al **2001** *Exp Eye Res*. 72:341). CB_1 gene (*Cnr1*)-deficient, ABH (ABH.*Cnr1*-/-) mice were generated by backcrossing, EAE-susceptible, ABH mice with CD1.*Cnr1*-/- mice (Ledent et al **1999** *Science* 283:401). This was undertaken as the CD1 mouse is largely refractory to EAE induction. Wildtype ABH mice developed a relapsing-remitting disease and slowly accumulated residual paresis following a number of relapses, when significant axonal loss and eventually spasticity developed. In contrast ABH.*Cnr1*-/- developed an aggressive clinical phenotype associated with a rapid accumulation of neurological deficits, including spasticity, even following a single attack as assessed by clinical signs and movement measured using an activity chamber. This was associated with significant axonal loss, consistent with the early and high expression of active, neuronal capase 3, an apoptotic death effector molecule. Wildtype ABH mice express this molecule notably in the relapsing phases of EAE after which neurological deficits are greatly increased. This indicates that CB_1 can mediate neuroprotective efffects during inflammatory autoimmune disease. During EAE, lesions can develop along the entire neuraxis of the CNS, whereas in EAU, induced in B10.RIII mice, lesions are focally restricted to the eye, here neural components of the retina such as photoreceptors are largely destroyed following one inflammatory insult. Daily administration of WIN55,212-2 (similar doses were ineffective at inhibiting the course of acute EAE), and notably THC produced a significant degree of retinal preservation, despite the presence of inflammatory lesions in all eyes. This suggests that CB_1 agonists, such as THC, could have some neuroprotective effect during MS, in addition to a modest CB_1-mediated immunosuppressive activity (demonstrated using high-dose THC in ABH.*Cnr1*-/- and ABH.*Cnr1*+/+ mice). Excitotoxic-mediated CNS cell death, occurs in many other experimental paradigms, such as ischaemia and head injury. We propose that similar mechanisms are chronically operating in inflammatory diseases of the CNS, which further suggest the potential neuroprotective role of the cannabinoids in these diseases.

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EFFECTS OF Δ^9-TETRAHYDROCANNABINOL IN ANIMAL MODELS OF HUNTINGTON'S DISEASE

Isabel Lastres-Becker, Emmanuel Brouillet[1], Nicolas Bizat[1], Frédéric Boyer[1], Onintza Sagredo, Philippe Hantraye[1], José A. Ramos, and Javier Fernández-Ruiz
Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Complutense, 28040 Madrid, Spain; [1]URA CEA CNRS 2210, Service Hospitalier Frédéric Joliot, DRM, DSV, Orsay Cedex, France

It has been suggested that the endocannabinoid system might be involved in the progression of neurodegeneration in Huntington's disease (HD). It has been also suggested that cannabinoids could be useful as neuroprotectant agents. In order to examine how a treatment with Δ^9-tetrahydrocannabinol (Δ^9-THC) could influence the neuronal degeneration in HD, we have used two different rat models of this disease. First, we used an acute model of HD generated by intrastriatal injections of malonate to Sprague-Dawley rats that produce neuronal death mainly via apoptosis and NMDA receptor activation. These rats were injected with Δ^9-THC (1 mg/kg and 10 mg/kg, i.p.) twice, 30 minutes before the intrastriatal injection of malonate and 2 hours later, and the animals were left for 48 hours before sacrifice. Our results showed that Δ^9-THC treatment increased the magnitude of the lesion as revealed the data of cytochrome oxidase (COX) and succinate dehydrogenase (SDH) activities. The second HD model used was generated by implantation of Alzet pumps containing 3-nitropropionic acid (3NP). This is a chronic model of HD in which the neuronal death mainly occurs by necrosis and it is not NMDA-mediated. 90 minutes after the Alzet pump implantation, rats were injected with Δ^9-THC (10 mg/kg) and the injection was repeated each 24 hours during a period of 4 days. The results showed that Δ^9-THC treatment was in this model neuroprotectant since it attenuated the reductions in both COX and SDH activities, as well as it produced a recovery in the neurotransmitter (dopamine and GABA) contents. In summary, our results are compatible with the idea that Δ^9-THC might be beneficial or harmful in neuronal injury models depending on the cell death pathway activated. So, Δ^9-THC increased the lesion in the acute model of HD where cell death occurs through apoptosis activation, but it decreased it in the chronic model where the death is mainly necrotic.

SCHEDULE-CONTROLLED BEHAVIOR IN CB_1 KNOCKOUT AND WILD TYPE MICE

Cassandra Y. Baskfield[1], Jenny L. Wiley[1], Anne M. Zimmer[2], Andreas Zimmer[2], and Billy R. Martin[1]
[1]Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA 23298; [2]Laboratory of Genetics and Cellular and Molecular Regulation, National Institute of Mental Health, Bethesda, MD 20892

Recombinant DNA techniques have made possible the generation of CB_1 knockout mice. These mice lack CB_1 cannabinoid receptors and are valuable tools in evaluating the CB_1 receptor-mediated effects of exogenous cannabinoids. CB_1 knockout mice also provide a unique opportunity to further investigate the role of CB_1 receptors in the effects of endogenous cannabinoids. In the present study, C57B1/6J mice devoid of CB_1 receptors (–/–) (N=18) and their wild type littermates (+/+) (N=12) were placed in standard mouse operant chambers and trained to lever press under a fixed-ratio (FR) 10 schedule of reinforcement. When stable rates of responding under the FR10 schedule had been established, cannabinoid and non-cannabinoids were administered in both groups. CB_1 knockout and wild type mice differed in learned schedule-controlled operant responding in response to the rate disruptive effects of the classical cannabinoid Δ^9-THC. Δ^9-THC produced a dose-dependent decrease in learned schedule-controlled responding in wild type mice. In addition, 30 mg/kg Δ^9-THC almost abolished responding in wild type mice. In contrast, the rate disruptive effects of Δ^9-THC were absent in CB_1 knockout mice. Δ^9-THC doses up to 56 mg/kg did not have significant effects on learned schedule-controlled responding in these genetically engineered mice. A more than ten-fold difference in the minimally effective dose of Δ^9-THC existed between the two lines. In contrast to results with the classical cannabinoid Δ^9-THC, CB_1 knockout and wild type mice did not differ in learned schedule-controlled responding in response to administration of methanandamide and 0-1812, synthetic endogenous cannabinoids. Further, SR141716A, the selective CB_1 cannabinoid antagonist, blocked the rate disruptive effects of Δ^9-THC in wild type mice, but did not attenuate the rate disruptive effects of methanandamide in either group. These results suggest that possible non-CB_1, non-CB_2 mechanisms of action for the rate disruptive effects of endogenous cannabinoids may exist. In addition, CB_1 knockout mice and their wild type littermates differed in response to ethanol. CB_1 knockout mice displayed greater decreases in schedule-controlled responding than their wild type littermates after administration of ethanol. Although unexpected and not readily explainable, these finding may stimulate further research investigating CB_1 receptors role in the behavioral effects of ethanol. Finally, the rate disruptive effects of the prototypic benzodiazepine diazepam did not produce differences in learned schedule-controlled operant responding in CB_1 knockout and wild type mice suggesting that diazepam's rate disruptive effects are not CB_1 receptor mediated.

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TOLERABILITY AND PHARMAOKINETICS OF ASCENDING DOSES OF Δ^9- THC-HEMISUCCINATE IN SUPPOSITORIES AND COMPARATIVE BIOAVAILABIITY WITH ORAL MARINOL®

Mahmoud A. ElSohly[1], Jennie Gwynn[2],
Peter Hylands[2], Norma Kellett[3], and Heather Bishop[3]
[1]National Center for Natural Products Research, School of Pharmacy, University of Mississippi, University, MS, 38677 USA and ElSohly Laboratories, Incorporated, Oxford, MS 38655 USA, [2]Oxford Natural Products plc, Charlbury, Oxfordshire, OX7 3EH, UK, and [3]Inveresk Research, Tranent, EH33 2NE, Scotland

Δ^9- THC-hemisuccinate (ONP-04) is a pro-drug of THC that hydrolyses rapidly to Δ^9- THC in plasma formulated in suppositories. This double-blind, placebo-controlled, randomized, parallel-group study evaluated the safety and tolerability of a range of doses of THC-hemisuccinate given as suppositories in 30 male subjects (Part I); followed by a comparative bioavailability study versus Marinol® (an oral formulation of THC) in 12 subjects (Part II).

In Part I, subjects received one of 5 ascending doses of THC-hemisuccinate (1.25 mg Eq, 2.5 mg Eq, 5 mg Eq, 10 mg Eq or 20 mg Eq of THC), or placebo. Blood samples were obtained before administration and 15min, 30min, 1h, 2h, 4h, 8h, 12h and 24h after each dose, and were analysed for THC, THC-COOH and 11-OH THC. As dose increased, AUC(0-∞) and C_{max}(obs) estimates for total THC increased broadly in proportion to dose. For THC-COOH, increases in estimates indicative of systemic exposure (AUC(0-∞), AUC(0-t) and C_{max}(obs)) were broadly dose proportional as dose increased to 5 mg Eq THC. As the dose increased further (*ie*, to 10 and 20 mg Eq THC), a subproportional increase was observed for THC-COOH from AUC(0-∞) measurements, although mean trends in C_{max}(obs) still appeared to increase in an approximately linear fashion throughout the dose range. C_{max}(obs) estimates for the active metabolite 11-OH-THC generally increased subproportionally with dose.

In Part II, subjects were dosed with a single rectal dose of THC-hemisuccinate of 10 mg Eq THC, and a single 10 mg oral dose of Marinol®. The order in which the treatments were administered was randomly allocated. Each dose was separated by a washout period of 14 days. For total THC, total systemic exposure (as indicated by AUC(0-∞) and AUC(0-t)) was considerably higher following rectal administration of THC-hemisuccinate 10 mg Eq THC compared with oral THC as Marinol® 10 mg. The relative bioavailability of total THC following administration of THC-hemisuccinate was on average 144.3% higher (by AUC(0-∞)) compared with administration of Marinol® . $T_{½}$el was longer following rectal administration of THC-hemisuccinate compared with oral Marinol®: mean half-life estimates of THC were 3.93h for ONP-04 (rectal dosing) compared with 1.14h for the orally given Marinol®. No serious adverse event was reported for any subject and there were no withdrawals because of an adverse event in either part of the study.

CANNABIS USE BY PATIENTS WITH CHRONIC NONCANCER PAIN: A PROSPECTIVE SURVEY

Mark Ware[1], Crystal Doyle[2], Ryan Woods[3], Mary Lynch[2], and John Clark[2]
[1]Pain Centre, McGill University Health Centre, Montreal, Quebec
[2]Pain Management Unit, Dalhousie University, Halifax, Nova Scotia
[3]Canadian HIV Trials Network, Vancouver, British Columbia

Chronic pain is one of the most common reasons for cannabis to be used medicinally. Little is known about the prevalence of such use among pain clinic populations, and such estimates would be useful in developing appropriate health education messages. Information on patterns of cannabis use among this population may also be useful in developing clinical trials of cannabis and cannabinoids for relief of chronic pain.

Over a six-week period in mid-2001, 209 subjects were recruited in a prospective anonymous cross-sectional survey. 72 (35%) subjects reported ever having used cannabis. Compared to never-users, cannabis ever-users were significantly younger (mean age 42y vs. 53yrs), were more likely to be male (51% vs. 31%), and were more likely to be tobacco users (63% vs. 20%).

32 (15%) subjects reported having used cannabis for pain relief (pain users), and 20 (10%) subjects were currently using cannabis for pain relief. 38 subjects denied using cannabis for pain relief (recreational users). Trauma and/or surgery as the cause of pain was significantly more common among pain users than recreational users (51% vs. 33% respectively). Pain users were more likely to have neck/upper body and myofascial pain than recreational users (68% & 65% vs. 42% & 30% respectively). The median duration of pain was similar in both groups.

There was a wide range of amounts and frequency of cannabis use. Of the 32 subjects who used cannabis for pain, 17 (53%) used four puffs or less at each dosing interval, 8 (25%) smoked a whole joint and 4 (12%) smoked more than one joint. 7 (22%) of these subjects used cannabis more than once daily, 5 (16%) used it daily, 8 (25%) used it weekly and 9 (28%) used it rarely. Pain, sleep and mood were the most commonly reported symptoms helped by cannabis use, and "high" and dry mouth were the most commonly reported side effects.

We conclude that cannabis use is prevalent among the chronic noncancer pain population, particularly among younger male subjects, and that the amounts used are variable but generally quite small, and often associated with tobacco use. Pain patients should be asked about cannabis use to facilitate health education and follow up, and to monitor for side effects and interactions with other medications. Clinical trials of cannabis and cannabinoids for chronic noncancer pain are warranted.

MEDICINAL CANNABIS EXTRACT IN CHRONIC PAIN: OVERALL RESULTS OF 29 "N OF 1" STUDIES (CBME-1)

William Notcutt, Mario Price, Cathy Sansom, Sue Simmons, and Cheryl Phillips
James Paget Hospital, Lowestoft Road, GREAT YARMOUTH, Norfolk, NR31 6LA, UK

Introduction

The results of 29 patients who have undertaken the study are presented.

Methods

The methodology of the study has been described in Poster 1. All patients had had a range of Pain Management previously but were currently inadequately pain controlled.

Results

Patients with Multiple Sclerosis, chronic back pain & sciatica after spinal surgery, other neuropathic pains and a myopathy have been recruited. The first 6 patients all had previous experience of cannabis and 5 were regular users. This gave us early information on effectiveness of the new materials via the sublingual route. . They were able to use the mixture of THC:CBD as escape medication to ensure that they did not return to using their previous cannabis. Subsequently, no patients have had cannabinoid escape medication, but have used their normal analgesics 29 patients started the 12 week study. 2 patients did not complete the study (1 suffered unacceptable side-effects within 2 weeks, and 1 could not cope with the study structure). 3 patients showed some initial benefit but this was not demonstrated in any of the parameters measured in the double blind placebo controlled period.

Benefits

The benefits were seen in many of the parameters studied and varied substantially between patients even within diagnostic groups. Improvements in pain, sleep, depression, activity and general health were the most important. 1 patient returned to skilled manual work and others continued a variety of activities and occupations. All the 24 patients who showed benefit have started a long-term safety extension study (see separate poster 3).

Side effects

3 patients experienced postural hypotension (1 vaso-vagal collapse) during acute titration. This was associated with excessive dosage in the early stages of the studies. Several patients noted mild dysphoria as they reached their individual ceiling dose. During regular usage, dry mouth was the most frequent side effect. Drowsiness, time distortion, high, dizziness, mild panic were also seen and most commonly associated with THC use.

Discussion

The extracts and the route of administration are effective. It is possible to titrate the sublingual CBME accurately and customize this for the individual patient. The variety of pain problems seen validated the study method we used. The 1-week periods were probably not long enough for some patients who could take several days to shown change in symptom intensity. This may reflect a prolonged pharmacological effect of the cannabinoid or a "resetting" of synaptic and neural activity.

[The results of a further 5 patients will be added]

CANNABIS MEDICINAL EXTRACTS (CME), INCLUDING CANNABIDIOL, ALLEVIATED NEUROGENIC SYMPTOMS IN PATIENTS WITH MULTIPLE SCLEROSIS AND SPINAL CORD INJURY

P.J. Robson[1], D.T. Wade[2], P.M. Makela[2], and H. House[2]
[1]Oxford University Department of Psychiatry, Warneford Hospital, Oxford OX3 7JX, UK
[2]Rivermead Rehabilitation Centre, Abingdon Road, Oxford OX1 4XD, UK
PJR also holds the post of Medical Director, GW Pharmaceuticals plc

Introduction: THC and other cannabinoids have been shown (Baker et al, **2000**) to ameliorate tremor and spasticity in a well-validated animal model of multiple sclerosis (MS). Anecdotal reports and uncontrolled observations in a small number of patients (Dunn & Davis, **1974**; Petro, **1980**; Clifford, **1983**; Consroe et al, **1986**; Meinck et al, **1989**; Breneissen et al, **1996**) suggest that cannabis can relieve a number of neurogenic symptoms associated with MS and spinal cord injury (SCI). Two small placebo-controlled studies (Petro & Ellenberger, **1981**; Ungerleider et al, **1987**) indicated that THC was superior to placebo in relieving spasticity. Standardised medicinal extracts from the cannabis plant (CME) may have advantages over single synthetic cannabinoids because of likely synergy between plant components.

Method: Plant extracts of delta-9-THC, cannabidiol (CBD), and a 1:1 mixture of THC and CBD were administered sublingually by pump-action spray. A series of 24 patients (MS-18; SCI-4; other-2) with at least one neurogenic symptom unresponsive to standard treatments were studied. Initial dosing was in clinic followed by self-titrated home dosing. After baseline measures and a two week run-in on THC:CBD 1:1 patients entered a randomised double-blind crossover comparison of the three CME and placebo with two-week treatment periods. For ethical reasons, initial patients had access to THC:CBD 1:1 as rescue medication throughout the crossover. Outcome was assessed by standardised measures of disability, mood and cognition, numerical scales at each assessment point, and visual analogue scales in a daily diary.

Results: Four patients withdrew due to adverse effects (AE) (hypotension, intoxication, ventricular ectopics unrelated to medication, sore mouth). Initially dosing was too rapid and was slowed for subsequent patients. Unequivocal alleviation of at least one target symptom (pain, spasticity, muscle spasms, bladder-related symptoms, tremor) by CME was seen in all of the remaining 20 patients. In subjects (n=12) who took little or no rescue medication, all three CME significantly reduced severity of spasticity and spasms, and frequency of nocturia in comparison with placebo. In addition, both THC and CBD significantly improved pain and stimulated appetite, while THC:CBD 1:1 significantly improved sleep. Intoxication occurred most frequently after THC. AE were predictable and generally well tolerated, and percentage of patients recording at least one AE during the crossover were as follows: CBD 33%, THC 60%, THC:CBD 1:1 30%, placebo 48%.

Conclusion: CME can benefit many neurologically-based symptoms including pain, spasticity, muscle spasms, lower urinary tract disturbance, and possibly ataxia. Unwanted effects are predictable and generally well tolerated. Larger scale studies are warranted in a variety of neurological conditions.

Acknowledgement: This study was supported by GW Pharmaceuticals

EFFICACY, SAFETY AND TOLERABILITY OF AN ORALLY ADMINISTERED CANNABIS EXTRACT IN THE TREATMENT OF SPASTICITY IN PATIENTS WITH MULTIPLE SCLEROSIS

C.Vaney[1], P. Jobin[1], F.Tschopp[1], M.Heinzel[2], and M. Schnelle[2]
[1]Neurologische Rehabilitations- and MS-Abteilung, Berner Klinik, Montana, Switzerland
[2]Institute for Oncological and Immunological Research, Berlin, Germany

Background: Patients reports, preclinical data and small clinical studies suggest that cannabis may alleviate muscle spasms, tremor, pain and bladder dysfunction associated with multiple sclerosis. We wanted to investigate the effect of an orally administered Cannabis sativa plant-extract on subject self–report and physician-administrated spasticity scales in a larger group of MS patients with persistent spasms not responding to other drugs.

Method: 57 patients were enrolled in a prospective, randomised, double-blind, placebo-controlled cross-over study. After randomisation to group A or B, patients received cannabis extract capsules (15 mg THC in 3 divided doses) for 15 days and then placebo for 10 days. These 2 periods were separated by a 5-day washout period and the drug treatment phase included an initial variable dose optimisation-finding period. Group B started with placebo first. Subject self-report scales included a daily spasm frequency scale and a symptoms diary. The physician-administrated scales (Ashworth Scale, Rivermead Mobility Index, the 3 composites of the MSFC, digit span, SF 36) were performed at the beginning and the end of each treatment period.

Findings: Whereas we did not observe an objective tone reduction in the drug group compared to placebo as rated by the Ashworth Score, but a statistically significant ($p<0.05$) reduction of spasms frequency occurred under plant extract treatment. Mobility as assessed by the RMI tended to improve more under drug. There wasn't any effect on tremor, sleep quality or micturition. Adverse events were qualitatively more severe during plant-extract treatment, but were quantitatively equal for both periods.

Interpretations: A Cannabis sativa plant extract might be beneficial to increase mobility and lower spasm frequency with tolerable side effects in patients with MS.

Acknowledgments: This study was supported by the Swiss Ministry of Health.

SHORT-TERM EFFECTS OF CANNABINOIDS IN PATIENTS WITH HIV-1 INFECTION

Donald Abrams[1], Roslyn Leiser, Joan Hilton, Starley Shade,
Tarek Elbeik, Francesca Aweeka, Neal Benowitz, Barry Bredt, Bradley Kosel,
Judith Aberg, Steven Deeks, Thomas Mitchell, Kathleen Mulligan,
Joseph McCune, and Morris Schambelan
University of California San Francisco, San Francisco, CA

Introduction: Widespread use of smoked marijuana prompted this study of its safety in patients with HIV-1 infection. Cannabinoids could impact on HIV RNA levels (viral load) by either an interaction with the immune system or the cytochrome P450 enzyme system altering metabolism of the protease inhibitor antiretroviral drugs.

Methods: A prospective randomized placebo-controlled 21day inpatient trial was conducted in the San Francisco General Hospital General Clinical Research Center. HIV+ patients with a stable viral load on a stable protease-inhibitor based antiviral regimen that contained either nelfinavir (NFV) or indinavir (IDV) were randomized to one of three treatment interventions- 3.95% tetrahydrocannabinol marijuana cigarette (M), dronabinol 2.5 mg (D) or placebo (P)- each three times daily before meals. Change in HIV RNA at 21 days was the primary endpoint with CD4+ cell counts, plasma levels of protease inhibitors, and testosterone levels evaluated as secondary safety parameters. Data for day 0 through day 21were analyzed using repeated measures. Changes in weight, appetite and body composition were also evaluated.

Results: Of 67 subjects enrolled, 21 were randomized to M, 25 to D and 21 to P. The majority were male (96%), people of color (52%) and over 40 years of age (67%). The HIV RNA level was <50 copies/mL for 37 (55%) at baseline and the median CD4+ cell count was 340/mm^3 (CD8+: 757/mm^3). Thirty subjects were on IDV 800mg q8h; 37 were on NFV 750mg tid. There were no statistically significant differences among the treatment groups by any of these baseline characteristics. Log_{10} HIV RNA levels declined in the marijuana (-0.19 log_{10}; p=0.13) and dronabinol (-0.24 log_{10}; p=0.06) arms compared to those on placebo. Eliminating 5 cannabinoid subjects who experienced significant declines in HIV RNA levels during the lead-in phase prior to commencing study drug, no change in HIV RNA levels were seen in any of the three arms. Change after 21 days in CD4+ T lymphocyte counts did not differ significantly in the M (+29 cells/mm^3; p=0.17) and D (+28 cells/mm^3; p=0.35) arms compared to P (+1 cell/mm^3). Cannabinoid recipients experienced CD8+ lymphocyte increases compared with the placebo group (M: +181 cells/mm^3; p=0.007; D: + 60 cells/mm^3; p=0.17; P: + 18 cells/mm^3). Although there was no statistically significant difference across arms, there was a 10% reduction in NFV AUC_8 (p=0.15) and a 15% reduction in the IDV AUC_8 (p=0.07) in the M group at day 14 compared to baseline. A non-significant decline in serum testosterone levels occurred in the cannabinoid recipients. Over the 21-day study, P recipients gained a median of 1.1 kg while the M and D subjects gained significantly more weight (M: +3.0kg; D: +3.2 kg), although the weight gain was predominantly fat.

Conclusions: Smoked marijuana has no significant short-term detrimental effects on standard safety parameters in HIV+ patients on a stable PI containing regimen.

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PROLONGED EFFECTS OF MARIJUANA ON MEN AND WOMEN

Alberta Springer, Karen Bolla, Ronald Herning,
Warren Better, Kimberly Tate, and Jean Cadet
Molecular Neuropsychiatry Section, National Institute
on Drug Abuse, Baltimore MD, 21224 USA

Marijuana remains the most commonly used illicit drug in the United States. According to the data from the 1998 NHSD, more than 72.0 million Americans 12 years of age and older have tried marijuana at least once in their lifetime, and almost 18.7 million had used marijuana in the past year. More than 120,000 people enter treatment per year for their primary marijuana addiction. Most problematic among young marijuana abusers is the view that marijuana is not harmful (Heyman et al., Pediatrics, **1999**,104, 982). Cognitive and psychiatric impairment has been documented with prolonged heavy marijuana use (Tunving et al., Psychiatry Res., **1986**, 17, 15; Pope et al., JAMA **1996**, 23, 521; Ehrenreich et al., Psychopharmacology, **1999**, 142, 295). A number of reports have suggested that habitual use of cannabis may alter cognitive functioning, particularly memory ability (Block et al., Psychopharmacology, **1993**, 110, 219; Rogers., et al., Psychopharmacology., **2000**, 151, 19). Forty marijuana abusers (32 male/8 female) and 51 control subjects (38 men/13 female) were studied. All subjects had no higher than a high school education. Before undergoing neuropsychological testing, all volunteers had undergone medical, neurological, psychological, and laboratory evaluations. All marijuana users met the DSM-III criteria for marijuana dependence or abuse using the Diagnostic Interview Schedule (Robins et al., NIMH Diagnostic Interview Schedule Version III-R, **1988**). Demographic information and drug use history information were obtained from the Addiction Severity Index (McLellan et al., NIDA, Treatment Research Reports, **1986**). An analysis of variance was used on the performance measures of the tests. The marijuana abusers performed more poorly than the control subjects on the Trail Tests (Trials A: $F(1,87)=0.44$, $p>0.05$; Trails B: $F(1,87)= 8.09$, $p> 0.05$), but not in the Stroop tests (Word: $F(1,87)= 0.90$, $p>0.05$; Color: $F(1.87)=0.41$, $p>0.05$; Interference: $F(1,87)= 3.42$, $p>0.05$; Composite: $F(1,87)= 4.71$, $p>0.05$). The number of errors on the Trail test did not differ between the groups (Trails A: $F(1,87) = 0.36$, $p>0.05$; Trails B: $F(1,87) = 1.35$, $p>0.05$). The reaction time was longer on the simple reaction time task for the marijuana group ($F(1,87)= 7.62$, $p>.01$); Choice reaction time did not differ: ($F(1,87) = 3.42$, $p > .05$). There were no impairments found for the Trails A or Trails B tests for the marijuana group. During the Stroop Test neurocognitive impairments were observed for the marijuana group. The marijuana group performed slower on the simple reaction time task, but not on the choice reaction time task. In this investigation subtle neurocognitive deficits were seen in attention, planning, mental flexibility, executive functioning, and psychomotor functioning. This study indicates that prolonged use of marijuana and other cannabis preparations may cause mild to moderate neurocognitive impairments.

EFFECTS OF PRENATAL MARIJUANA EXPOSURE ON SCHOOL ACHIEVEMENT AT AGE 10

Lidush Goldschmidt, Nancy Day, and Gale Richardson
Program in Epidemiology, Western Psychiatric Institute and Clinic,
University of Pittsburgh, Pittsburgh, PA 15213, USA

Research about the effects of prenatal marijuana exposure on children's school achievement is scarce. In one of the few studies on the topic, the Ottawa Prenatal Prospective Study found that there was no relationship between in utero exposure to cannabis and reading and language scores of 9- to 12- year-old children (Fried et al., **1996**). We examined the association between prenatal marijuana exposure and academic achievement at age 10, after controlling for home environment, socioeconomic status, and other maternal substance use. In addition, the mediating effect of the child's depression on this relationship was tested. The strengths of the present study include a large sample size and a representative sample of a low income population.

This is a prospective study of women who were recruited from a prenatal clinic. They were interviewed at the end of their 1st, 2nd, and 3rd trimesters of pregnancy about their marijuana and other substance use. Half of the women were Caucasian and half were African-American. Follow-ups occurred at 8 and 18 months, 3, 6,10, 14, and 16 years. The 10-year assessment of achievement included the Wide Range Achievement Test (WRAT-R) reading, spelling, and arithmetic scores, the Peabody Individual Achievement Test (PIAT-R) reading comprehension score, and the teacher's overall ranking of the child's academic performance. Child depressive symptoms were measured by the Children's Depression Inventory (Kovacs, **1992**). The analysis included 606 mother-child dyads.

The majority of the women decreased their use of marijuana as their pregnancy progressed. During the 1st, 2nd, and 3rd trimesters, 14%, 5%, and 5% of the women were heavy users (≥ 1 joint/day), respectively. Using regression analyses, 1st trimester heavy use of cannabis was significantly related to WRAT-R scores and to teacher's ranking of the child's performance, after adjusting for other relevant covariates. Reading and spelling scores of the heavily exposed children were about 3 points lower than the non-heavily exposed children. Heavy exposure was also significantly related to depression. After controlling for the child's depression, the relationship between marijuana exposure and achievement was no longer significant. The finding that child depression mediates the relationship between prenatal marijuana exposure and academic performance has important implications for the type of intervention which should be provided to reduce the adverse effects of drug exposure.

SIMILARITIES AND DISSIMILARITIES IN COGNITIVE PERFORMANCE OF ADOLESCENTS EXPOSED PRENATALLY TO MARIJUANA AND CIGARETTES

Peter A. Fried, Barbara Watkinson, and Robert Gray
Department of Psychology, Carleton University, Ottawa, Ontario K1S 5B6, Canada

The Ottawa Prenatal Prospective Study (OPPS) is an ongoing longitudinal project that was initiated in 1978. It has examined the neurobehavioral and developmental effects of prenatal exposure to marijuana and cigarettes in a low-risk cohort from birth through adolescence. Since the early eighties, these children have been the subject of numerous reports examining the association between prenatal exposure to marijuana or cigarettes and a large number of cognitive and behavioral domains. The present report is an examination of cognitive performance in 13-16 year OPPS offspring. Several relatively consistent trends have been noted within the cognitive sphere when these children were younger. Prenatal marijuana exposure does not appear to impact on overall IQ but does have a negative association with tasks/problems requiring complex visual analysis. On the other hand, prenatal cigarette exposure appears to have a negative impact on IQ and tasks requiring auditory processing and verbal comprehension. Similar findings have been reported in other cohorts.

The outcomes presented in this report are derived from a large neuropsychological battery administered to the OPPS adolescents. The assessment instruments used to evaluate cognition included measures of achievement, general intelligence, memory and facets of executive functioning. Within each of these domains visual and auditory competence was examined.

The major findings can be summarized as follows: prenatal marijuana exposure did not have an impact on IQ but was negatively associated with tasks requiring visual memory, analysis and integration; prenatal cigarette exposure was negatively associated with IQ and auditory memory. These observations remained statistically significant after controlling for a number of possibly confounding factors including the subjects' own use of either cigarettes or marijuana. The findings are consistent with and extend the observations in the cognitive domain made in this sample when they were younger and serve to emphasize the differential and long-lasting consequences of in utero exposure to marijuana and cigarettes within this sphere of functioning.

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ATTENUATION OF CANNABINOID WITHDRAWAL IN DOUBLE MU AND DELTA OPIOID RECEPTOR KNOCKMOUT MICE

R. Maldonado[1], A. Castañé[1], B.L. Keiffer[2], P. Robledo[1]
[1]Univ Pompeu Fabra, Barceloa, Spain
[2]IGBMC, Illkirch, France

Several studies have shown functional relationships between the endogenous cannabinoid and opioid systems. However, acute delta[9]-tetrahydrocannabinol (THC) pharmacological responses and physical dependence were not modified in knock-out mice with single delection of mu, delta or kappa opioid receptors. To further investigate the neurobiological basis of cannabinoid dependence, we have evaluated THC responses in double mu and delta opioid receptor knock-out mice. Antinociception and hypolocomotion induced by acute THC administration remain unaffected whereas the acute hypothermic effects were slightly attenuated in these double mutants. During chronic THC treatment, knock-out mice developed slower tolerance to the hypothermic effects but the development of tolerance to antinociceptive and hypolocomotor effects was almost unaffected. The rewarding properties of THC were abolished in knock-out mice. Interestingly, the somatic manifestations of THC withdrawal were also significantly attenuated in mutant mice, suggesting that a cooperative action of mu and delta opioid receptors is required for the entire expression of THC dependence.

INTERRELATIONSHIP OF CANNABINOID AND OPIOID DEPENDENCE MECHANISMS

Billy R Martin, and Aron H Lichtman
Department of Pharmacology and Toxicology, Medical College of Virginia Campus,
Virginia Commonwealth University, Richmond, VA

Opioids and cannabinoids are two distinct classes of compounds with unique pharmacological properties. However, they do share some common pharmacological traits, one of which is antinociception. Moreover, it is now well documented that opioids and cannabinoids interact, at least in part, to produce antinociception. Historically, there has been considerable interest in establishing commonalties in the dependence properties of both cannabinoids and opioids. In recent times, several laboratories have demonstrated at least some form of cross-dependence between opioids and cannabinoids. In our own laboratory, we demonstrated that precipitated THC withdrawal was ameliorated in mu opioid receptor knockout mice and failed to occur in mice devoid of CB_1 cannabinoid receptors. Morphine decreased withdrawal signs in THC-dependent mice. Morphine dependence was attenuated in CB_1 receptor knockout mice. Acute treatment of THC attenuated some morphine dependence signs. Acute administration of specific mu (morphine), delta (SNC 80), and kappa (U50,488 and enadoline) agonists ameliorated precipitated withdrawal in THC-dependent mice. Taken together these findings implicate a role for opioid receptor subtypes in the modulation of cannabinoid dependence. These findings taken implicate a reciprocal relationship between the cannabinoid and opioid systems in dependence.

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FUNCTIONAL INTERACTIONS BETWEEN CANNABINOIDS AND OPIOIDS IN ANIMAL MODELS OF SELF-ADMINISTRATION

W. Fratta, G. Cossu, and L. Fattore
Department of Neuroscience, University of Cagliari, Cagliari, Italy

Despite the fact that cannabis is one of the oldest abused drugs, it has been difficult for a long time to demonstrate cannabinoid rewarding effects in laboratory animals. Recently, we have developed two models of cannabinoid intravenous self-administration: a model of acute intravenous self-administration in drug-naive mice and a model of chronic intravenous self-administration in Long-Evans rats. We have shown that cannabinoids are self-administered by mice and rats in a way similar to other drugs of abuse. Furthermore, we have hypothesized a functional interaction between opioid and cannabinoid self-administration. In fact, CB_1 receptor antagonist SR141716A antagonized morphine self-administration whereas cannabinoid self-administration was antagonized by the opioid antagonist naloxone. We have also shown that mice lacking the CB_1 receptor do not self-administer morphine and morphine is unable to stimulate dopamine release in nucleus accumbens of these mice. These data strongly support the hypothesis of a mutual functional interaction between cannabinoid and opioid systems for the expression of rewarding effects of both opioids and cannabinoids.

ENDOCANNABINOIDS AND APPETITE: POSSIBLE INTERACTIONS WITH OTHER NEUROTRANSMITTER SYSTEMS

George Kunos[1], D. Osei-Hyiaman[1], M. Palkovits[2], M.J. Kuhar[3], K. Mackie[4], and A.W. Bannon
[1]National Institute on Alcohol Abuse and Alcoholism,
[2]National Institute of Health, Bethesda, MD 20892,
[3]Emory University, Atlanta, GA; [4]University of Washington,
Seattle, WA; [5]Amgen Co., Thousand Oaks, CA

Previoius studies have indicated that endocannabinoids acting via CB_1 receptors contribute to the hunger-induced increase in food intake in mice. Since CB_1 receptors in the brain are presynaptically located, a possible mechanism of a CB_1-mediated orexigenic effect could involve presynaptic inhibition of the release of a tonically active anorexigenic mediator, such as a-MSH, CRH or CART. SR141716 (3 mg/kg ip) did not affect food intake in food-restricted CART knockout mice, whereas it significantly reduced food intake in their wild-type littermates. Of brain regions implicated in appetite control, abundant CART immunostaining was found in various hypothalamic areas including the arcuate, paraventricular and dorsomedial nuclei, as well as in the amygdala and the nucleus accumbens, with very low levels in the cingulated cortex. In contrast, CB_1 was minimally detectable in the hypothalamus and nucleus accumbens, but high levels were found in the cingulated cortex and amygadala. These findings suggest that CART may be a downstream mediator involved in the orexigenic effect of endocannabinoids, although the site and exact nature of their possible interaction remains to be identified.

WIN55212-2, A CANNABINOID AGONIST, AND U-50,488H, A KAPPA OPIOID AGONIST, PRODUCE SYNERGISTIC HYPOTHERMIA

S.M. Rawls, R.J. Tallarida, E.B. Geller, and M.W. Adler
Center for Substance Abuse Research, Temple University,
School of Medicine, Philadelphia, PA, USA

Opioids and cannabinoids share a number of physiological actions, including thermoregulation. Kappa and mu receptors mediate the hypothermic and hyperthermic effects, respectively, of opioids in rats. Cannabinoid agonists produce hypothermia through CB_1 receptors. Given the functional similarities between opioids and cannabinoids, we investigated their interaction on body temperature in male SD rats. WIN55212-2 (WIN; 1-10 mg/kg, i.m.), a selective cannabinoid, or U-50,488H (U50; 5-20 mg/kg, i.m.), a selective kappa opioid, evoked dose-dependent hypothermia. Pretreat-ment (24 hr) with nor-BNI (1mg/kg, s.c.), a selective kappa antagonist, attenuated the response to WIN, suggesting that kappa receptors contribute to cannabinoid-induced hypothermia. Neither SR141716, a selective CB_1, nor SR144528, a selective CB_2, antagonist altered the effect of U-50 (10mg/kg). Using the dose-response relations of WIN and U50, we studied a fixed-ratio combination (0.525 U50: 0.475 WIN). The observed effects exceeded the expected (additive) for each dose pair, thereby demonstrating a synergistic interaction for the two drugs.

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CB_1 ANTAGONIST SR141716A-INDUCED BLOCKADE OF MU-RECEPTOR DESENSITIZATION IN THE SH-SY5Y CELL LINE

H.Z. Ozsoy, and K.M. Standifer
Department of Pharmacological and Pharmaceutical Sciences,
University of Houston, Houston, TX 77024

Interactions between opioid and cannabinoid systems regarding tolerance and dependence have been demonstrated in in vivo studies. Cellular mechanisms underlying these functional interactions are unknown. Chronic morphine (MS) treatment (24 h) produces a significant desensitization of mu receptor-mediated inhibition of forskolin-stimulated cAMP accumulation in SH-SY5Y neuroblastoma cells that endogenously express mu and CB_1 receptors. Protein kinase C (PKC), PKC-alpha and PKC-epsilon, were upregulated by chronic MS treatment. Also chronic MS treatment decreased G-protein (Gi and Go) solubility in sodium cholate. Concominant use of the CB_1 antagonist SR141716A (SR) with MS blocked MS-induced mu receptor desensitization. In addition SR blocked the MS-induced PKC-alpha and PKC-epsilon upregulation, whereas it did not block the MS-induced Gi and Go solubility shift in sodium cholate. Our data suggests that the CB_1 antagonist, SR141716A, interferes with MS-induced changes in PKC levels that may contribute to its ability to block mu receptor desensitization. >

THE EFFECTS OF ORAL ADMINISTRATION OF Δ^9-THC ON MORPHINE TOLERANCE AND PHYSICAL DEPENDENCE

Diana L. Cichewicz, and Sandra P. Welch
Department of Pharmacology and Toxicology,
Virginia Commonwealth University, Richmond, VA 23298 USA

Recent reports from our laboratory indicate that a low dose of delta-9 tetrahydrocannabinol (Δ^9-THC) enhances the potency of oral opioids in a mouse acute pain model (Cichewicz et al., *J Pharmacol Exp Ther*, 289: 859-867, **1999**). The analgesic effect of a low dose combination of these drugs is similar to that of a high dose of morphine. Due to the common occurrence of tolerance in human patients treated with morphine for chronic pain, we were eager to determine whether a Δ^9-THC/morphine combination would provide an alternate pain therapy without the development of tolerance or dependence. Thus, we hypothesized that 1) morphine tolerance would develop in mice after 7 days of oral administration; 2) co-administration of Δ^9-THC during tolerance development would diminish or eliminate morphine tolerance and physical dependence; and 3) a combination of low doses of Δ^9-THC and morphine would provide effective analgesia without tolerance or physical dependence. Morphine tolerance was produced in ICR mice by administration of high doses of morphine p.o. twice daily for 7 days. This paradigm resulted in a 3-fold shift to the right of the morphine dose-response curve. When Δ^9-THC at a low dose of 20 mg/kg p.o. was administered along with each morphine dose for 7 days, there was no significant difference in the ED_{50} of morphine as compared to vehicle-treated mice. Thus, the co-administration of Δ^9-THC was effective in preventing the development of morphine tolerance. Similarly, there was no difference in the dose-response curves for morphine in mice treated with a combination of low doses of both Δ^9-THC and morphine (20 mg/kg p.o.) for 7 days as compared to vehicle-treated mice. These results suggest that chronic administration of a low dose-combination may provide analgesia similar to that required by patients on long-term morphine without the development of tolerance to the drug. We also examined the propensity of this combination to inhibit the development of physical dependence to morphine. After chronic treatment, mice were challenged with 1 mg/kg naloxone s.c. to precipitate opioid withdrawal and observed for a 10-minute period for jumping behavior and forepaw tremors. Morphine-tolerant mice showed platform jumping behavior with a mean jumping latency of 5-6 minutes, while mice treated with Δ^9-THC and high doses of morphine showed a reduction in jumping and an increased jumping latency. The chronic administration of a low dose combination of Δ^9-THC and morphine (20 mg/kg) completely attenuated the expression of withdrawal signs. These data indicate that combination treatment with Δ^9-THC and morphine may be a successful analgesic therapy while prolonging or preventing the development of physical dependence to morphine.

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THE CSXXFP MOTIF IS AN IMPORTANT STRUCTURAL ELEMENT OF THE SECOND EXTRACELLULAR LOOP IN THE CANNABINOID CB_1 AND CB_2 RECEPTORS

Judy Barnett-Norris, Dow P. Hurst, and Patricia H. Reggio
Department of Chemistry, Kennesaw State University, Kennesaw, GA 30144 USA

Although the cannabinoid receptors are classified as members of the rhodopsin (Rho) family of G-protein coupled receptors (GPCRs), CB_1 and CB_2 differ from Rho in several important ways. One of these is the E-2 loop length and the space occupied by this loop. The CB E-2 loops are shorter than that of Rho (19 residues-CB_1; 17 residues-CB_2; 27 residues Rho). In Rho, a disulfide bridge between C3.25 and a Cys residue in the E-2 loop causes this loop to dip down from the top of TMH 4 into the binding pocket toward TMH 3 and then return to the top of TMH 5. *The E-2 loop in Rho, therefore, occupies a significant amount of space above (and partly inside) the TMH bundle.* The CB receptors lack the C3.25. However, there is a Cys residue at the extracellular end of TMH 4, and a Cys near the middle of the E2 loop in the CB receptors that may form a disulfide bridge. *As the result of these important E-2 loop differences between Rho and the CB receptors, the position of the E-2 loop with respect to the binding site crevice in CB_1/CB_2 around TMHs 3-4-5 is likely to be quite different from that in Rho.*

The CB receptors share a CSXXFP motif in their E-2 loops. A search of GPCR sequences for the CSXXFP E-2 loop motif, combined with no Cys at 3.25, but a D/ERY motif at the intracellular end of TMH 3, resulted in only two hits, CB_1 and CB_2. However, a search for the more generalized CSXXXP motif along with the other requirements resulted in a match with one other GPCR (EDG-1) and three orphan receptors (rCNL3, 6-7 and ARG16, see Song et al. *FEBS Lett*. 351:375-379, **1994**). In order to evaluate whether this conserved motif has any structural consequences, we undertook a study, first of the isolated E-2 loop peptides and then of each loop in the context of its TMH bundle.

The CB_1 (NCEKLQSVCSDIFPHIDET) and CB_2 (CPRPCSELFPLIPND) E-2 loops were simulated using the Conformational Memories method (Guarnieri et al. *J. Am. Chem. Soc. 118*: 5580, **1996**) as implemented in CHARMM with an implicit solvent model for water to simulate the loop environment. Ninety-six torsions for CB_1 E-2 and seventy-four torsions for CB_2 E-2 were varied. The final structures (100 for each loop) were clustered using the backbone atoms of the CSXXFP motif. For CB_1 there were four clusters of 34,19,17,and 11 members and for CB2 there were three clusters of 29, 28, and 10 members. When the cluster representative structures for each of the loops were combined (N=7) and clustered using the backbone atoms of CSXXFP, three main structural families were identified, each of which contained both CB_1 and CB_2 loops and differed only in the PSI angles for the XX residues of CSXXFP. **These results show that the CSXXFP motif introduces *distinct turns common to both the CB_1 and CB_2 E-2 peptides* with the PSI angles for the XX residues of CSXXFP creating the distinction between the three clusters.**

To further investigate the conformations of the E-2 loops, a Monte Carlo/Simulated Annealing protocol combined with a loop and disulfide bridge closure algorithm is currently being used on both the CB_1 and CB_2 E-2 loops in the presence of their TMH bundles in order to determine E-2 loop relative placement on the top of the TMH bundle.

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1-METHOXY-, 1-DEOXY-11-HYDROXY- AND 11-HYDROXY-1-METHOXY-Δ^8-TETRAHYDROCANNABINOLS: NEW SELECTIVE LIGANDS FOR THE CB_2 RECEPTOR

Simon M. Bushell[1], John W. Huffman[1], John R. A. Miller[1],
Jenny L. Wiley[2], and Billy R. Martin[2]
[1]Department of Chemistry, Clemson University, Clemson, SC 29634-0973, USA
[2]Department of Pharmacology and Toxicology, Medical College of Virginia Campus,
Virginia Commonwealth University, Richmond, VA 23298-0613, USA

The complex pharmacological effects of cannabinoids are considered to be mediated through at least two G-protein coupled, transmembrane receptors. One of these, designated as CB_1 is found predominantly in the central nervous system and is responsible for most of the overt pharmacological effects of cannabinoids. A second receptor, designated CB_2, was originally identified from macrophages present in the spleen, and is expressed primarily in the periphery. Although it has been known for some time that cannabinoids are involved in immunomodulation, the discovery that CB_2 receptors are expressed primarily in the immune system led to the suggestion that the CB_2 receptor was responsible for the immunomodulatory effects of cannabinoids. This suggestion has recently been confirmed by the observation that such effects are absent in CB_2 receptor knockout mice. Furthermore, recent studies have now highlighted other beneficial pharmacological effects specific to the CB_2 receptor. These effects include the discovery that the CB_2 selective ligand, JWH-133, is effective in reducing spasticity in the mouse model of multiple sclerosis. More pertinently, the same CB_2 selective ligand inhibits the *in vivo* growth of glioma tumors. Other effects modulated by the CB_2 receptor include peripheral antinociception and, at least in part, the antitumor properties of ajulemic acid.

This presentation will detail the design, synthesis and pharmacology of three new series of CB_2 selective ligands, based on the traditional cannabinoid nucleus. These are the 1-methoxy-, 1-deoxy-11-hydroxy- and 11-hydroxy-1-methoxy-Δ^8-THC moieties, each bearing a 3-(1',1'-dimethylalkyl) pendant sidechain of between two and seven carbon atoms in length. Of these, the 1-methoxy-3-(1',1'-dimethylhexyl)-Δ^8-THC, JWH-229, has effectively no affinity for the CB_1 receptor (K_i = 3134 ± 110 nM) but has high affinity for the CB_2 (K_i = 18 ± 2 nM).

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EFFECTS OF WIN55212-2 AND NORADRENALINE ON THE MOUSE VAS DEFERENS ARE ATTENUATED BY (–)-CANNABIDIOL

Susan J. Craib, Adèle Thomas, Ruth A. Ross, and Roger G. Pertwee
Department of Biomedical Sciences, Institute of Medical Sciences,
University of Aberdeen, Aberdeen AB25 2ZD

(–)-Cannabidiol (CBD) has been reported to attenuate several *in vivo* effects of Δ^9-tetrahydrocannabinol in human subjects (Karniol *et al.,* **1974**). In the present experiments, we investigated whether CBD alters the ability of the CB_1/CB_2 agonist, WIN55212-2, to inhibit evoked contractions of the mouse isolated vas deferens, a tissue in which prejunctional CB_1 receptors are thought to mediate inhibition of evoked release of the contractile transmitters, noradrenaline (NA) and adenosine 5'-triphosphate (ATP).

WIN55212-2 and CBD were dissolved in DMSO and other drugs in saline or in saline/ascorbic acid (NA bitartrate). CBD was added 30 min before the other drugs. Additional experimental details and the method used to calculate a value for K_B are described elsewhere (Pertwee *et al.,* **1995**; Ross *et al.,* **2001**).

At 0.32, 1.0, 3.2, 6 and 10 μM but not at 0.1 μM, CBD attenuated the ability of WIN55212-2 to inhibit electrically-evoked contractions, producing concentration-related dextral shifts in the log concentration response curve of this agonist that did not deviate from parallelism and were not accompanied by any changes in E_{max}. Data analysis yielded a Schild plot with a slope that did not deviate significantly from unity (1.2; 95% confidence limits of 0.8 & 1.6) and a mean K_B value for CBD of 120 nM, well below its reported K_i values for displacing ligands from CB_1 or CB_2 binding sites: 4.35 and 2.86 μM respectively (Showalter *et al.,* **1996**) and >10 μM (Bisogno *et al.,* **2001**). The amplitude of evoked contractions increased significantly in the presence of CBD at concentrations of 0.32 μM and above but not in the presence of DMSO. Contractile responses of the vas deferens to 10 μM NA were decreased by 1 and 10 μM but not by 0.1 μM CBD. However, 10 μM CBD did not affect the ability of the stable ATP analogue, γ-methyleneATP, to induce contractions of this tissue.

These experiments indicate that CBD can enhance electrically-evoked contractions and attenuate contractile responses to NA in the mouse vas deferens. They also show that CBD can antagonize WIN55212-2 in a competitive, surmountable manner by acting on sites that are unlikely to be CB_1 or CB_2 receptors or to be located postjunctionally.

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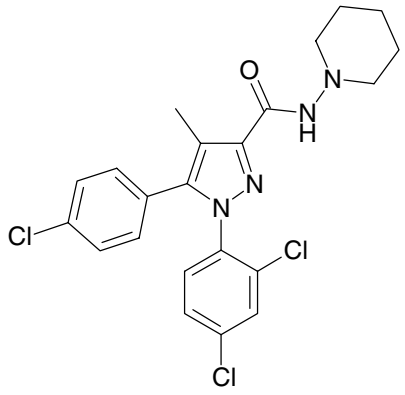
NOVEL RING-CONSTRAINED ANALOGS OF THE CB_1 ANTAGONIST SR141716

Maria E. Francisco[1], Herbert H. Seltzman[1], Jason P. Burgess[1], Gregory S. Bailey[1], Clifford George[2], Anne F. Gilliam[1], and Brian F. Thomas[1]
[1]Research Triangle Institute, Research Triangle Park, NC 27709, USA
[2]Naval Research Laboratory, Washington, DC 20375, USA

The biaryl pyrazole SR141716 (**1**) is a potent, CB_1-selective cannabinoid antagonist. While many analogs of this compound have been synthesized, very few of the modifications have involved replacement of the pyrazole ring. In order to investigate the structure-activity relationships of the central ring moiety, while keeping the spatial relationship of the 2,4-dichlorophenyl, 4-chlorophenyl, methyl and hydrazide moieties intact, phototransposition studies were conducted on **1**. The phototranspostion strategy involves rearrangement of the pyrazole to an imidazole ring through exposure to a 450-W high-pressure mercury lamp for 150 min. This approach has been described with other pyrazole ring systems (Pavlik et al., *J. Org. Chem.* **1991**, *56*, 6313; *J. Am. Chem. Soc.*, **1993**, *115*, 7645). While in other phototransposition studies, many products are frequently generated, in this case, a single reaction product was observed. However, when this compound was isolated by high-pressure liquid chromatography, the desired imidazole ring structure was not apparent. Instead, a novel ring-constrained compound was synthesized. Its structure was elucidated by spectroscopic and crystallographic means. This compound has reasonably high affinity for the CB_1 receptor (K_i = 48.0 ± 2.7 nM). Its affinity at the CB_2 receptor and its ability to affect GTP-γ-[^{35}S] is currently being determined. Further examination has indicated that ring modification from pyrazole to imidazole via phototransposition is not likely to be achieved with the substituents present in SR141716. However, synthesis of further ring-constrained analogs of SR141716 appears to be feasible, and this approach is currently being investigated.



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CHANGES IN GENE EXPRESSION IN RESPONSE TO CANNABINOID AND AMINOALKYLINDOLE AGONISTS

Allyn C. Howlett, Porsha L. Carter, Jenelle D. Jones, and Kent E. Vrana
Neuroscience/Drug Abuse Research Program, J. L. Chambers Biomedical/Biotechnology Research Institute, North Carolina Central University, Durham, NC 27707 USA; Department of Physiology and Pharmacology, Wake Forest University School of Medicine, Winston-Salem, NC 27157 USA

Changes in gene expression patterns due to chronic drug treatment were studied in cloned N18TG2 neuroblastoma cells that were originally derived from a mouse spinal cord tumor. These cells express CB_1 cannabinoid receptors and respond to agonists by activating G protein signal transduction pathways. We hypothesized that gene expression would be modified in response to chronic treatment with cannabinoid and aminoalkylindole classes of CB_1 receptor agonists.

Neuroblastoma cells were treated for forty-eight hours with 1) cannabinoid agonist, CP55244, 2) aminoalklylindole agonist, WIN55212-2, or 3) vehicle control. The RNA was isolated using TriReagent and RNA samples were quantified and visualized by gel electrophoresis. cDNA samples were labeled with phosphorus-32 deoxynucleotides, and hybridized to Clontech mouse cDNA microarrays. Images of the hybridization pattern were scanned with a Phosphorimager, and data were analyzed using Clontech Atlas Software.

Comparison of microarray densities showed that mRNA for soluble guanylate cyclase beta 1 subunit, sentrin, pre-synaptic density protein-95, and putative protein tyrosine phosphatase were up-regulated by both CB_1 receptor agonists. Continuing studies are examining the influence of chronic drug treatment on protein synthesis rates, with particular emphasis on the guanylate cyclase pathway.

Some discrepancies were found in the microarray density patterns exhibited by cells treated with the two CB_1 receptor agonists. For example, Ran GTPase activating protein (GAP) mRNA appeared to be decreased in response to CP55244 but increased in response to WIN55212-2. CP55244 increased cellular mRNA for the protein axin, and WIN55212-2 increased cellular mRNA for the protein "frizzled homolog 6". The finding that the aminoalkylindole WIN55212-2 produced a pattern of gene expression somewhat different from the cannabinoid CP55244 might suggest the presence of two different receptors for the two ligands. An alternative interpretation is that the CB_1 receptor can transduce long-term changes via different signal transduction pathways depending upon the agonist ligand that stimulates the receptor. These two possibilities are being investigated.

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NUCLEAR MAGNETIC RESONANCE STUDIES OF CANNABINOID RECEPTOR SECOND EXTRACELLULAR LOOP PEPTIDES CONTAINING A CONSERVED CSXXXP SEQUENCE IN THE ABSENCE AND PRESENCE OF SDS

Anna M. Hutchings, Vicky L. H. Bevilacqua, John H. Brown,
Travis P. Albright, Dow P. Hurst, and Patricia H. Reggio
Department of Chemistry and Biochemistry,
Kennesaw State University, Kennesaw, GA, 30144-5591, USA

Recent mutagenesis and chimera studies have implicated the second extracellular loops (E2) of CB_1 and CB_2 in ligand binding. As a contribution toward understanding the role of E2 in the interactions of CB_1 and CB_2 with cannabinoids, we have initiated structure studies on two E2 peptide analogues in the absence and presence of SDS using nuclear magnetic resonance (NMR) spectroscopy. The peptide analogues have the sequences EKLQSVSSDIFPHIDET (CB_1 analogue) and SP--RPSSELFPLIPND (CB_2 analogue), with a Cys-Ser substitution at position 5. Sequential assignments were carried out using Total Correlation Spectroscopy (TOCSY) and Nuclear Overhauser Effect Spectroscopy (NOESY) or Rotating Frame Nuclear Overhauser Effect Spectroscopy (ROESY) spectra[1] for samples of 1-10 mM (pH range 5.60-5.69). X-PLOR 3.1[2] was employed to calculate and minimize 50 random starting structures, followed by simulated annealing with heating to 2000K and cooling to 100K using NOE data as constraints.

Based on alpha proton chemical shifts, CB_2-E2 and CB_1-E2 have random coil conformations in an aqueous environment. The N-terminal half (residues E_1-S_8) of CB_1-E2 in SDS micelles contains an alpha-helical stretch. CB_2-E2 in the presence of SDS consists of at least two equally populated stable conformations, each having alpha proton chemical shifts consistent with random coil conformations. Preliminary simulated annealing calculations suggest that the E-2 peptides in both the absence and presence of SDS contain a well-defined region that includes the CSXXXP sequence shared by the cannabinoid receptors and several orphan receptors. The precise conformation of the CSXXXP region depends on the peptide and the solvent system.

1) J. Evans, "Biomolecular NMR Spectroscopy," 1995, Oxford University Press, NY; 2) Axel T. Brünger, "X-PLOR version 3.1," (1992) Yale University Press, New Haven.

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PYRROLE-BASED NON-TRADITIONAL CANNABINOIDS

Lea W. Knight, John W. Huffman, and Matthew L. Isherwood
Department of Chemistry, Clemson University, Clemson, SC 29634-0973, USA

Following the discovery of the aminoalkylindoles by the Sterling-Winthrop group, it was concluded that these compounds interact with the same receptor as classical cannabinoids, such as Δ^9-THC. This led to the proposal of at least two different pharmacophores which align these structurally different ligands with the CB_1 receptor. One proposed alignment (Huffman *et al. Bioorg. Med. Chem. Lett.* **1994**, *4*, 563) overlays the naphthoyl carbonyl of the aminoalkylindoles with the phenolic hydroxyl of traditional cannabinoids. The ring system is oriented such that the indole nitrogen corresponds to C-1' of the traditional cannabinoid side chain. The naphthalene ring of the indole corresponds to the cycloalkyl portion of the THC nucleus and the aminoalkyl moiety corresponds to the alkyl side chain. This alignment has been used as a model to prepare a number of potent cannabimimetic indoles. An additional suggestion which explains the interaction of the aminoalkylindoles with the CB_1 receptor focuses on aromatic stacking interactions between the receptor and the ligands (Bramblett and Reggio, **1995**, *Symposium on the Cannabinoids,* International Cannabinoid Research Society; Burlington, VT, **1995**, 16).

Based on the assumption that only the pyrrole ring portion of the indole moiety should be necessary for the interaction of the ligand with the receptor, a series of 3-(1-naphthoyl)-*N*-alkylpyrroles were synthesized and evaluated for receptor affinity. These compounds exhibit significantly less affinity for the CB_1 receptor than the corresponding indoles and are less potent *in vivo* (Lainton *et al. Tetrahedron Lett.* **1995**, *36*, 1401.) On the assumption that aromatic stacking interactions are important in the interaction of cannabimimetic pyrroles with the receptor, a second set of pyrroles was synthesized which bear a phenyl ring at the 2-position. This series shows significantly increased receptor affinity, especially with *N*-pentyl and *N*-hexyl substituents (K_i = 11.6 nM and K_i = 9.4 nM, respectively). To investigate the effect of placing substituted aryl groups at the 2-position and their effect upon receptor affinity, additional 2-arylpyrroles have been prepared and their affinities for the CB_1 receptor determined.

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AROMATIC RESIDUES IN HELIXES 3-5-6 OF CB_1 PROVIDE SPECIFIC INTERACTION SITES FOR WIN55,212-2 AND SR141716A

Sean D. McAllister[1], Dow P. Hurst[2], Kurt Buehner[2], Judy B. Norris[2], Patricia H. Reggio[2], and Mary E. Abood[1]
[1]Forbes Norris ALS Research Center, California Pacific Medical Center, San Francisco, CA 94115, [2]Department of Chemistry, Kennesaw State University, Kennesaw, GA 30144

Mutation studies have suggested a separation of the binding site for aminoalklyindoles (AAIs) from other classes of cannabinoid agonists. In addition, SR antagonists and AAIs may share a binding region, consistent with their structural similarities. Our modeling studies indicate that aromatic-aromatic stacking interactions in CB_1 receptor helixes 3-4-5-6 may form this region. The following aromatic residues have been identified as important to the binding of AAIs: F200(3.36), W255(4.64), and W279(5.43). For SR141716A, F200(3.36), W279(5.43) and W356(6.48) are identified as important ligand interaction sites. While F189(3.25) is not part of the identified aromatic clusters, modeling studies suggest that this residue forms the "ceiling" of the anandamide binding pocket in CB_1.

In order to study the importance of these aromatic residues, we employed site directed mutagenesis to receptor cDNA, and each appropriate amino acid was individually mutated to an alanine. Ligand binding analysis was carried out in stable cell lines expressing the mutant receptors. Each receptor was also individually expressed in Xenopus oocytes along with two G-protein gated inwardly rectifying potassium channels (GIRK1 and GIRK4) for functional analysis.

Ligand binding analysis revealed a significant loss of affinity for both WIN55,212 (WIN) and SR141716A but not CP55,940 and anandamide (AEA) at F200A(3.36), W279A(5.43), and W356A(6.48) when compared to wild-type CB_1. At the F189A(3.25) receptor, ligand affinity was only reduced in the presence of AEA. In agreement with previous studies, our data suggested W255A(4.64) was not expressed on the cell surface.

The potency and efficacy of AEA but not WIN at F189(3.25) was significantly reduced which equated well with the binding data. In the presence of 1 μM drug, the mutant F200A(3.36) could only produce a maximum response of 24, 15 and 9 % with WIN, AEA, and CP55940, respectively. These results suggest this amino acid was not only an interaction site for WIN and SR141716A but was also important participant in receptor activation. In agreement with the binding data the potency of WIN at W279A(5.43) was significantly reduced. However, both the potency and efficacy of AEA was also reduced at W279A(5.43). These findings indicate that this residue may also play a role in receptor activation since the binding affinity for AEA at W279A(5.43) was unchanged in transfected HEK cells. The response to WIN and AEA at W356A(6.48) was not different from wild-type CB_1.

These results demonstrate the importance of aromatic residues in helixes 3-4-5-6 of CB_1 for ligand recognition and signal transduction. This data also further supports the hypothesis that AAIs and SR antagonists may share common interaction sites at CB_1.

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MOLECULAR BASIS FOR CANNABINOID RECEPTOR SUB-TYPE SELECTIVITY OF AM411 AND AM756

Cindy Tartal[1], John Steed[1], Dow Hurst[1], Dai Lu[2, 3],
Alex Makriyannis[2, 3], and Patricia Reggio[1]
[1]Department of Chemistry, Kennesaw State University, Kennesaw, GA 30144, USA
[2]Center for Drug Discovery, [3]Department of Pharmaceutical Sciences
University of Connecticut, Storrs, CT 06269,USA

AM411 and AM756 are classical cannabinoid ligands in which the C-3 alkyl side chain has been replaced with an adamantyl or an alkenyl-adamantyl side chain. These ligands are CB sub-type selective (AM411: CB_1 K_i=5.9 nM, CB_2 K_i=60.0 nM; AM756: CB_1 K_i=48.6 nM, CB_2 K_i=8.9 nM). In order to investigate the molecular basis for the sub-type selectivity of these compounds, AM1 Conformational Analyses and Unique Volume Map calculations were performed for these two compounds. The structures of AM411 and AM756 were built in the Spartan molecular modeling program (V4.1.1; Wavefunction, Inc. Irvine, CA). AM1 conformational searches were performed for each side chain rotateable bond (12-fold rotation).

AM1 conformational searches identified four conformers of AM411 that differed in the orientation of the adamantyl ring. In Conf 1 (global min), the adamantyl ring was oriented such that the C1' – C2' bond is in the plane of the phenyl ring and parallel with the C4-H bond (C2-C3-C1'-C2' = -179.9^o). . In Conf 2 ($\Delta(\Delta Hf)$=0.03 kcal/mol), the adamantyl ring was oriented such that the C1'-C2' bond is in the plane of the phenyl ring (C2-C3-C1'-C2' = 0.7^o) and the C2' methylene bridge hydrogens are staggered with respect to H of C2. In Conformers 3 and 4, which were 1.09 and 1.14 kcal/mole above the global min, the adamantyl ring was rotated such that the C1'-C2' bond is out of the phenyl ring plane (Conf 3, C2-C3-C1'-C2' = 153.1^o; Conf 4,C2-C3-C1'-C2' = –152.3^o).

Six conformers were identified for AM756. All of these conformers placed the adamantyl ring either in the top or bottom face of the molecule. The $\Delta(\Delta Hf)$ values in kcals/mol and C2-C3-C1''-C2' torsion angle values for these conformers were found to be - Conf. 1: 0.0 kcal/mol, 134.8 ^{o} ; Conf 2: 0.04, -133.2 ^{o} ; Conf 3: 0.02, 50.6 ^{o} ;Conf 4: 0.07, -51.0 ^{o} ;Conf 5: 0.63, 97.0 ^{o} ;and Conf 6: 0.66, -85.1 ^{o} .

In order to illustrate the key conformational differences between AM756, and AM411, we used a modification of the Active Analog Approach (J.R. Sufrin et al. Mol. Pharmacol. **1981**, *19*, 307) to calculate the volume of space that is **unique** to the CB_2 selective analog, AM756. Map results suggested that there would be a difference in the requirements for adamantyl ring interaction with a hydrophobic pocket at each CB receptor sub-type. The CB_2 selective analog, AM 756 could occupy space above and below the aromatic ring; while the space occupied by the adamantyl ring in the CB_1 selective analog, AM 411, remained to the side of the aromatic ring. Receptor docking studies in R* models of CB_1/CB_2 suggested that selectivity is related to the size of the hydrophobic pocket available in the transmembrane helix (TMH) 3-5-6 region. While the adamantyl side chain of AM 756 could be accommodated in this region of CB_1, the CB_2 binding site provided a pocket in the TMH 3-5-6 region that could accommodate the adamantly ring, cupping it with hydrophobic residues with which it could form more productive hydrophobic interactions.

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CANNABIDIOL ATTENUATES RESPONSES OF THE MOUSE VAS DEFERENS TO CANNABINOID AND NON-CANNABINOID RECEPTOR AGONISTS

Adèle Thomas, Susan J. Craib, Ruth A. Ross, and Roger G. Pertwee,
Department of Biomedical Sciences, Institute of Medical Sciences,
University of Aberdeen, Aberdeen AB25 2ZD

CB_1 receptor agonists inhibit electrically-evoked contractions of the mouse isolated vas deferens, most probably by acting through prejunctional CB_1 receptors to decrease the release of contractile transmitters. Recently we obtained evidence that (–)-cannabidiol (CBD) attenuates the ability of the CB_1/CB_2 receptor agonist, WIN55212-2, to inhibit evoked contractions of this tissue by acting on sites that are unlikely to be CB_1 or CB_2 receptors or to be located postjunctionally (Craib *et al.,* unpublished). In the present experiments, we investigated if CBD also antagonizes certain other cannabinoid or non-cannabinoid inhibitors of evoked contractions of the mouse isolated vas deferens: the CB_1/CB_2 receptor agonists, CP55940 and anandamide (AEA), the VR1 agonist, capsaicin, the α_2 adrenoceptor agonist, clonidine, and the μ opioid receptor agonist, [D-Ala^2, NMePhe^4, Gly-ol]-enkephalin (DAMGO). Drugs were dissolved in DMSO (CBD, CP55940, AEA and capsaicin), in a 50% aqueous solution of DMSO (v/v) (WIN55212-2) or in distilled water or saline. CBD was added 30 min before all other drugs. Further experimental details are described elsewhere (Pertwee *et al.,* **1995**; Ross *et al.,* **2001**).

At 10 μM, CBD significantly attenuated the ability of CP55940, capsaicin, clonidine and DAMGO to inhibit electrically-evoked contractions of the vas deferens, producing a dextral shift in the log concentration response curve of each of these agonists that did not deviate from parallelism. The mean dextral shifts, with 95% confidence limits shown in brackets were 294.8 (111.9 & 858.7), 13.0 (7.0 & 25.5), 6.9 (4.9 & 10.0) and 2.8 (1.8 & 4.2) respectively. Corresponding values for 10 μM CBD against WIN55212-2 were 88.6 (48.8 & 158.4). AEA was also significantly antagonized by 10 μM CBD. However, no value for the dextral shift in its log concentration response curve could be calculated from our data as in the presence of 10 μM CBD, AEA produced only slight inhibition of evoked contractions even at the highest concentration used (10 μM). At 3.16 μM, CBD produced a 19.8 fold mean dextral shift in the log concentration response curve of AEA with 95% confidence limits of 9.7 & 46.4. Corresponding values for 3.16 μM CBD against WIN55212-2 were 28.3 (13.2 & 59.5).

In conclusion, although CBD attenuated the responses to all the twitch inhibitors that we investigated, the degree of antagonism it produced was agonist-dependent. It was most effective against CP55940, WIN55212-2 and AEA, less effective against capsaicin and clonidine and least effective against DAMGO. The responses to CP55940, AEA and WIN55212-2 were equally attenuated by CBD as were the responses to capsaicin and clonidine. Further experiments are now underway to establish the mechanism(s) underlying these effects of CBD.

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Pertwee, R.G. *et al.* (1995) *Eur. J. Pharmacol.* **284,** 241-247.
Ross, R.A. *et al.* (2001) *Br. J. Pharmacol.* **132,** 631-640.

CONFORMATIONAL MEMORIES AND CURVATURE ANALYSES FOR ENDOCANNABINOID INTERACTION WITH THE ANANDAMIDE TRANSPORTER AND THE FATTY ACID AMIDOHYDROLASE

Hassimi Traore[1], Kurt Buehner[2],Judy Norris[2], Diane Lynch[2], and Patricia Reggio[2]
[1]Department ofChemistry, University of Wisconsin-Whitewater,
Whitewater, WI 53190, USA; [2]Department of Chemistry,
Kennesaw State University, Kennesaw, GA 30144, USA

SAR for endocannabinoid interaction with the anandamide transporter and fatty acid amidohydrolase reveals a divergence in acyl chain requirements. While both the transporter and the amidohydrolase recognize C18 to C20 fatty acid acyl chains, their requirements for degree of unsaturation differ. In order to probe the acyl chain conformational requirements for interaction with the transporter, the method of Conformational Memories (CM; Guarnieri et al. *J. Am. Chem. Soc*. **1996**, *118*, 5580) was used to study the following ethanolamides: 18:0 (IC_{50} >100 μM), 18:1 Δ^9 cis (10.5 ± 1.2 μM), 18:1 Δ^9 trans (>100μM), 19:2 $\Delta^{8,11}$ (18.3 ± 4.2 μM), 20:1 Δ^{11} (14.1 ± 1.2 μM), 20:2 $\Delta^{9,12}$ (10.6 ± 1.5 μM), 20:3 $\Delta^{8,11,14}$ (13.0 ± 1.2 μM), and 20:4 $\Delta^{5,8,11,14}$ (anandamide, 15.1±3.0 μM) (D.Piomelli et al. *Proc. Natl. Acad. Sci USA* **1999**, *96*:5802).

CM results for the inactive 18:0 and 18:1 Δ^9 trans analogs resulted only in families of extended conformers. CM results for other analogs studied resulted in extended and U-shaped families of conformers. The average radius of curvature of each U-shaped family was calculated using a curvature analysis method, adapted from Kumar and Bansal (*Biophys. J.* **1996**, *71*, 1574) that was originally designed for helix analysis. To fit the acyl chains to a circle, a subset of atoms, C4 to C18 was chosen. The average radius of curvature, r (in the C-4 to C-18 region) of the U-shaped family of conformers was found to be : 20:1 Δ^{11}, r=11.69 ± 3.78Å; 19:2 $\Delta^{8,11}$, r= 8.14 ± 0.50Å; 20:2 $\Delta^{9,12}$, r=6.20 ± 0.46Å; 20:3 $\Delta^{8,11,14}$, r=4.35 ± 0.30 Å; 20:4 $\Delta^{5,8,11,14}$, r= 3.98 ± 0.25Å. ***These results suggest that the transporter recognizes only curved/U-shaped conformers and that among such curved conformers, the average radius of curvature must be between 11.69 Å and 3.98 Å, with the highest affinity for the lower radius of curvature.***

In order to probe the acyl chain conformational requirements for hydrolysis of fatty acid ethanolamides at FAAH (F. Desarnaud et al. *J.Biol.Chem.* **1995,** *270*, 6030), CM analyses of the following ethanolamides were performed: 18:3 $\Delta^{6,9,12}$ (45% hydrolysis), 20:1 Δ^{11} (2% hydrolysis), 20:2 $\Delta^{11,14}$ (35% hydrolysis), 20:3 $\Delta^{8,11,14}$ (35% hydrolysis) and 20:4 $\Delta^{5,8,11,14}$ (100% hydrolysis). CM results for FAAH indicate that each analog has an extended and a curved/U-shaped family. The average radius of curvature, r (in the C-4 to C-18 region) of the U-shaped family of conformers was found to be : 20:1 Δ^{11} r=11.69 ± 3.78Å; 20:2 $\Delta^{11,14}$, r= 6.40 ± 0.50Å ; 18:3 $\Delta^{6,9,12}$, r=4.22 ± 0.50Å ; 20:3 $\Delta^{8,11,14}$, r= 4.35 ± 0.30Å; 20:4 $\Delta^{5,8,11,14}$, r=3.98 ± 0.25Å**.** ***It is clear here that the radius of curvature decreases with increasing number of double bonds. These results also suggest that radius of curvature requirements for FAAH are more stringent, with the enzyme showing significant hydrolysis for analogs with average radii of curvature between 6.40 and 3.98 Å.***

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SYNTHESIS AND EVALUATION OF NEW DERIVATIVES OF PALMITOYLETHANOLAMIDE AS INHIBITORS OF ANANDAMIDE HYDROLYSIS

Séverine Vandevoorde[1], Kent-Olov Jonsson[2], Christopher J. Fowler[2], and Didier M. Lambert
[1]Catholic University of Louvain, Medicinal Chemistry and Radiopharmacy unit, 1200 Brussels, Belgium; [2]Departments of pharmacology and Clinical Neuroscience, Umeå University, Sweden

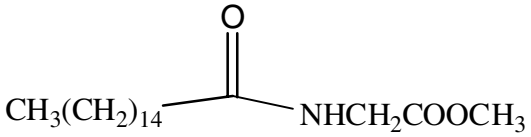
It is now well established that the endogenous fatty acid amide anandamide (AEA) has, as a result of its actions upon cannabinoid and vanilloid receptors, a number of interesting pharmacological properties including effects on nociception, memory processes, lung functions, spasticity and cell proliferation. However, its therapeutic use is limited because AEA is metabolised by the fatty acid amide hydrolase (FAAH).

Several FAAH inhibitors have been described, often based on the structure of AEA itself. However, in this case, the possibility that the compounds will interact directly with CB exists. Our alternative approach is to prevent the metabolism of AEA with compounds related to palmitoylethanolamide (PEA), since this fatty acid amide, although a substrate for FAAH, is inactive at cannabinoid receptors.[1]

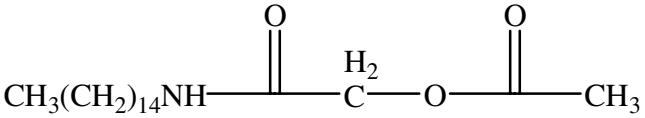
We synthesized new derivatives of PEA, keeping the C16 long chain of palmitic acid and modifying the « head » of the molecule : 17 alkylated-, 9 aromatic-, 5 halogenated-palmitic amides and 2 new derivatives of oleic acid (oleylethylamide and oleylisopropylamide) were prepared.

With the exception of oleyl derivatives, our compounds did not inhibit [^{3}H]CP 55,940 or [^{3}H]WIN55,212-2 binding in CHO-CB_1 and CHO-CB_2 cells, respectively.

The ability of our new derivatives to inhibit FAAH-catalysed hydrolysis of [^{3}H] AEA has been investigated using brain homogenates as source of FAAH. In the alkylamide family, palmitoylethylamide and palmitoylallylamide were found inhibitors of FAAH with pI50 of 5.45 and 5.47 respectively. Halogenated derivatives exhibit high p50 values but somewhat low percentages of maximal inhibition. Amides containing aromatic moieties were less potent inhibitors of AEA metabolism. Compounds containing amide and ester bonds, L55 and L58, showed nice pI50 values of 4.99 and 5.08, respectively.



L 55



L 58

[1] K.O. Jonsson, S. Vandevoorde, D.M. Lambert, G. Tiger and C. Fowler, *Br. J. Pharmacol.*, **133**, 1263-1275 (**2001**)

CYSTEINE 89 (2.59) IN THE SECOND TRANSMEMBRANE DOMAIN OF HUMAN CB_2 RECEPTOR IS EXPOSED IN THE BINDING CREVICE

Rundong Zhang, and Zhao-Hui Song
Department of Pharmacology and Toxicology
University of Louisville, Louisville, KY 40292, USA

Cysteine engineering, in combination with specific affinity labeling, are powerful tools to gain structural information of proteins, such as folding patterns, ligand binding sites, and conformational changes. As a first step to study the human CB_2 cannabinoid receptor using cysteine substitutions, the current study was carried out to identify the native cysteine residues that are exposed in the binding crevice of CB_2.

Using the method of site-directed mutagenesis, five cysteines in the transmembrane domains of CB_2 were mutated to serines one at a time. These mutant receptors, C40(1.39)S, C89(2.59)S, C257(6.47)S, C284(7.38)S and C288(7.42)S, were transfected into and stably expressed in HEK293 cells. Radioligand binding assays were performed to detect the susceptibility of specific [^{3}H]-HU243 binding to MTS reagent modification.

In our preliminary studies, methanethiosulfonate (MTS) derivatives were found to inhibit specific [^{3}H]-HU243 binding to wild-type CB_2, with a rank order of potency of MTS ethylammonium (MTSEA) > MTS ethyltrimethylammonium (MTSET) > MTS ethylsulfonate (MTSES). Four out of the five mutant receptors exhibited specific [^{3}H]-HU243 binding, with K_d values in the nanomolar range. However, CB_2C40(1.39)S had no detectable specific [^{3}H]-HU243 binding. The cysteine to serine mutation at position 2.59 of CB_2 drastically reduced the susceptibility of specific [^{3}H]-HU243 binding to MTS reagent modification. In contrast, the susceptibility of specific [^{3}H]-HU243 binding to MTS reagent modification remained intact in other mutant receptors.

Two conclusions can be made from the current study. 1. The specific binding of [^{3}H]-HU243 to wild-type CB_2 is susceptible to modification by MTS reagents. 2. C89(2.59), a residue at the upper portion of the second transmembrane domain of CB_2, is exposed in the binding crevice. In the future, the CB_2C89(2.59)S mutant receptor will serve as a background for the studies of other residues in the bindiig crevice of CB_2 using the substituted cysteine accessibility method.

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DIFFERENTIAL SENSITIVITIES OF ENDOCANNABINOIDS AND VANILLOIDS

Babatunde E. Akinshola[1], and Emmanuel S. Onaivi[2, 3]
[1]Howard University College of Medicine, Washington, DC, 20059,
[2]William Paterson University, Wayne, NJ 07470, and
[3]NIDA Intramural Program, National Institutes of Health, Baltimore, MD 21224

There is growing evidence for the existence of an endocannabinoid physiological control system (EPCS) which has been implicated in the treatment of pain, whereas the vanilloid receptor system (VRS) is involved in the sensory perception of pain. In this study, the effects of endocannabinoid and vanilloid agonists were compared on kainate activated AMPA receptor subunits in *Xenopus* oocytes using the two-electrode voltage clamp, in-vitro. The order of potency of inhibition of receptor current were arvanil > 2-AG > olvanil > methanandamide and 2-AG > arvanil > methanandamide > olvanil at the GluR1 and GluR3 respectively. The *in-vitro* data suggest similar roles for the EPCS and VRS in long term potentiation. *In-vivo*, the effects of cannabinoid receptor (*Cnr*) agonist and CB_1 antagonist, SR141716 in the mouse model of withdrawal aversions from cocaine, alcohol and diazepam was evaluated. The blockade of the behavioral aversions by the CB_1 antagonists following withdrawal from chronic administration from cocaine, alcohol and diazepam support some role for this EPCS in natural reward mechanism that warrants further investigation.

REGIONAL DIFFERENCES IN NALOXONE MODULATION OF Δ^9-THC – INDUCED C-FOS EXPRESSION IN RATS

Kelly Allen[1], Iain McGregor, Glenn Hunt, Malini Singhs[1], and Paul Mallet[1]
School of Psychology, University of Sydney, NSW 2006, Australia
[1]School of Psychology, University of New England, Armidale NSW 2351, Australia

Behavioral and pharmacological research shows extensive interplay between the cannabinoid and opioid neurochemical systems of the brain. For example, the opioid receptor antagonist, naloxone has been found to inhibit cannabinoid-induced activation of reward pathways, cannabinoid self-administration, cannabinoid-induced hyperphagia and cannabinoid antinociception. Here we sought to more rigorously characterise the neural basis of such effects. To this end we compared *c-fos* expression in groups of Wistar rats treated with vehicle, Δ^9-THC (10 mg/kg), naloxone (10 mg/kg) or Δ^9-THC and naloxone in combination. Locomotor activity was greatly depressed in both Δ^9-THC treatment groups and moderately inhibited in rats given naloxone alone. Naloxone inhibited Δ^9-THC-induced *c-fos* immunoreactivity in several key brain regions including the ventral tegmental area, islands of Calleja, central striatum, ventromedial and dorsomedial hypothalamus and ventrolateral periaqueductal grey. Conversely, naloxone potentiated THC-induced *c-fos* expression in regions of the extended amygdala associated with stress, including the central nucleus of the amygdala, bed nucleus of the stria terminalis and the paraventricular nucleus of the thalamus. Naloxone had no effect on Δ^9-THC-induced *c-fos* expression in the paraventricular nucleus of the hypothalamus, Edinger-Westphal nucleus, or nucleus accumbens core. These findings complement earlier results suggesting mediation of cannabinoid-induced analgesia, hyperphagia and reward by the opioid system. The ability of naloxone to inhibit Δ^9-THC *c-fos* expression in appetite related areas of the hypothalamus may explain the inhibitory effect of this drug on Δ^9-THC-induced hyperphagia while the similar effect in the ventrolateral PAG may explain why naloxone attenuates THC-induced analgesia.

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THE ROLE OF THE ENDOCANNABINOID SYSTEM IN BEHAVIOURAL AND PHYSIOLOGICAL RESPONSES TO RESTRAINT STRESS IN RATS

Jonathon Arnold, Hitoshi Sataka, and Rosemarie Einstein
Department of Pharmacology, University of Sydney, Sydney,
New South Wales 2006, Australia

The present study assesses the role of the endocannabinoid system in behavioural and physiological responses to restraint stress in rats. Male Wistar Kyoto rats were administered either the cannabinoid CB_1 receptor antagonist, AM281 (1 mg/kg i.v.) or vehicle. At this dose, AM281 effectively blocked cannabinoid CB_1 receptors as pretreatment with it reduced catalepsy promoted by the cannabinoid receptor agonist CP55,940 (400 µg/kg i.p.). After AM281 administration, rats were subjected to restraint stress or were placed back in their home cage for 1 hour in both conditions. This provided four experimental groups: AM281 + restraint; vehicle + restraint; AM281 + control; and vehicle + control. Immediately after restraint or the control procedure rats were either placed in the elevated plus maze (EPM) for 10 minutes to assess anxiety-related behaviour (n = 10), or blood was collected to determine plasma levels of corticosterone using a radioimmunoassay (n = 5). Behavioural testing showed that restraint stress decreased exploration of the open arms, which is consistent with an increase in anxiety-related behaviour. Surprisingly, AM281 pretreatment did not reverse this increase in anxiety-related behaviour as measured by the EPM. Furthermore, AM281 had no effect on anxiety-related behaviours in control animals. Restraint stressed animals showed significantly greater plasma corticosterone levels than control animals. Interestingly, AM281 pretreatment reversed this restraint stress-induced rise in plasma corticosterone levels. In control animals AM281 did not increase plasma corticosterone levels. These results suggest that antagonism of cannabinoid CB_1 receptors reverses restraint stress-induced increases in plasma levels of corticosterone, but does not reverse restraint stress-induced increases in anxiety-related behaviour.

CANNABINOID AGONISTS AND $G_{I/O}$-MEDIATED CELLULAR SIGNAL TRANSDUCTION MECHANISMS

Abdel-Azim Assi, Skyla Carney, LaTronya McCollum, Derek Norford, and Allyn Howlett
Neuroscience/Drug Abuse Research Program, JL Chambers Biomedical/Biotechnology Research Institute, North Carolina Central University, Durham, NC 27707 USA

It is well known that CB_1 cannabinoid receptors in the brain are coupled to pertussis toxin-sensitive G proteins of the G_i/G_o family. These G proteins may signal through multiple effector mechanisms. CB_1 cannabinoid receptors are expressed on N18TG2 neuroblastoma and C6 glioma cells. These studies sought to determine which signal transduction pathways may be activated by these G proteins.

Extracellular signal-regulated kinases 1 and 2 (ERK1/2) are a family of kinases that play an important role in cell proliferation and differentiation. N18TG2 and C6 glioma cells were serum-starved (12-24 h) and treated with cannabinoid receptor antagonist SR141716 prior to stimulation. Studying the time-course for such stimulation revealed that the maximum effect was obtained 2 minutes of treatment with different CB_1 agonists. Treatment of cells with CP55940, WIN55212-2 or methanandamide produced a concentration-dependent phosphorylation of ERK as quantitated by densitometry of bands on Western blots of cell lysates. The potency ratios were: CP55940 > WIN55212-2 > methanandamide. Pretreating cells with 100 ng/ml pertussis toxin attenuated the cannabinoid agonist-induced ERK activation, implicating Gi and/or Go in this signal transduction pathway.

Cannabinoid drugs have been shown to increase nitric oxide (NO) synthesis in a number of cells and tissues. We examined N18TG2 and C6 cells for their ability to produce NO as detected using 4-amino-5-methylamino-2', 7'-difluorofluorescein diacetate (DAF-FM) in intact cells. CB_1 cannabinoid agonists CP55940 and methanandamide stimulated NO production in both cell lines within minutes at 1 micromolar concentrations. Studies are ongoing to determine which G proteins may be involved in this response.

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EVIDENCE FOR NOVEL CANNABINOID RECEPTORS IN RAT BRAIN

Chris Breivogel, and Bhavita Parekh
Campbell University School of Pharmacy, Buies Creek, NC 27506 USA

Previous studies have indicated the presence of non-CB_1 G-protein-coupled receptors in brain using CB_1 knock-out mice (DiMarzo et al, **1999**; Breivogel, et al **2001**; Hanus et al, **2001**). The present study investigated the activity of these receptors in rat brain by determining CB_1 antagonist-insensitive activation of G-proteins by various cannabinoid ligands. Antagonism of the stimulation of [^{35}S]GTPγS binding by WIN55212-2 and CP55940 was measured in membranes from rat cerebellum and cortex using maximally effective concentrations of each agonist in the presence of various concentrations of each antagonist. In both cortex and cerebellar membranes, stimulation by CP55940 was completely blocked by SR141716A or AM251. In contrast, even in the presence of maximally effective concentrations of SR141716A or AM251, 30% of the stimulation produces by WIN55212-2 remained in cerebellar membranes and 50% of this activity remained in cortex membranes. Moreover, the same concentrations of these antagonists assayed in the absence of agonists had no significant effects on [^{35}S]GTPγS binding. K_i values were approximately 0.7 ± 0.2 nM for each antagonist versus either agonist, consistent with antagonism of CB_1-mediated activity.

Analysis of the stimulation of [^{35}S]GTPγS binding by various concentrations of WIN55212-2 in the presence and absence of 1 μM SR141716A was used to determine the potency and confirm the relative efficacy of WIN55212-2 at the unknown receptor. These experiments indicated that WIN55212-2 exhibited approximately half (47%) of the maximal stimulation of [^{35}S]GTPγS binding in the presence (43% stimulation) as in the absence (91% stimulation) of SR141716A. Comparison of the fitting of the data to one-site versus two-site model indicated that WIN55212-2 stimulated [^{35}S]GTPγS binding with two apparent potencies in the absence, and only one potency in the presence of SR141716A. The potency of WIN55212-2 in the presence of SR141716A (EC_{50} = 5.1 μM) approximately matched the low potency site measured in the absence of SR141716A (EC_{50} = 0.4 nM and 0.9 μM). These data indicated that the CB_1 antagonist blocked all of the high potency CB_1-mediated activity of WIN55212-2 leaving only the lower-potency activity of the unidentified site. These data extend the previous results that indicated that WIN55212-2 (and anandamide) activated a non-CB_1 G-protein-coupled receptor in mouse brain, but CP55940 and the other ligands tested did not.

Acknowledgements: V. Di Marzo, C.S. Breivogel, Q. Tao, D.T. Bridgen, R. Razdan, A.M. Zimmer, A. Zimmer and B.R. Martin, (**2000**), Journal of Neurochemistry 75 (6): 2434-2444. C.S. Breivogel, G. Griffin, A.M. Zimmer, A. Zimmer, V. Di Marzo and B.R. Martin, (**2001**) Molecular Pharmacology 60 (1): 155-163., N. Hajos, C. Ledent, T.F. Freund (**2001**) Neuroscience 106 (1): 1-4.

FUNCTIONAL ASSAYS FOR CANNABINOID CB_1 AND CB_2 RECEPTORS BY HETEROLOGOUS EXPRESSION IN *SACCHAROMYCES CEREVISIAE*

Andrew J Brown[1], Simon Dowell[1], Carl Haslam[1], Alan Wise[1], Nick Clayton[2], Alan Naylor[2], Celestine O'Shaughnessy[2], Diane Cousens[1], and Shelagh Wilson[3]
[1]Discovery Research, GlaxoSmithKline, Gunnels Wood Road, Stevenage, SG1 2NY; [2]Neurology CEDD, GlaxoSmithKline, New Frontiers Science Park, Harlow, Essex, CM19 5AW, UK; [3]Discovery Research, GlaxoSmithKline, New Frontiers Science Park, as above

The budding yeast *Saccharomyces cerevisiae* is now widely used as an expression system to provide functional assays for mammalian G protein-coupled receptors (GPCRs). In this approach, receptors are expressed in genetically modified yeast strains in which agonism results in activation of an endogenous signal transduction pathway and induction of reporter genes. Receptor coupling is promoted by the presence of chimeric yeast/mammalian Gα subunits. Yeast GPCR assays are readily amenable to high-throughput approaches to identify novel ligands, owing to their simplicity and the ease of yeast cell culture.

The cannabinoid CB_1 and CB_2 receptors represent highly tractable drug targets in therapeutic areas ranging from obesity to asthma, MS, pain, IBS, and nasal allergy. We have developed yeast-based functional assays for both CB_1 and CB_2. Human CB_2 couples with high efficiency (median effective concentration (EC_{50}); 2.7 ± 0.97 nM, n=7), using the standard agonist HU210. This is comparable to values obtained in [^{35}S]-GTPγS binding assays (EC_{50}; 2.5 ± 0.33 nM) using membranes containing CB_2 and mammalian G-proteins, from transfected Sf9 cells. Coupling of human CB_1 is slightly less efficient, (EC_{50}; 26.7 ± 1.3 nM; n=5), relative to reported values of 2.5 ± 0.96 nM in GTPγS binding. Antagonists such as AM251 and AM630 have their expected specificities for CB_1 and CB_2, respectively.

A feature of this system is that receptors are often less well coupled than in recombinant mammalian assays, primarily due to absence of the cognate wild-type G protein heterotrimer. The recently described anandamide analogues, arachidonylcyclopropylamide (ACPA) and arachidonyl-2-chloroethylamide (ACEA), both behave as partial agonists at CB_1 in yeast. This is in keeping with their effects in tissue-based assays, but contrasts with stably transfected CHO cells where both are full agonists [Hillard *et al.*, (**1999**) JPET 289:1427-1433]. One unexpected finding was that both ACPA and ACEA are relatively efficacious at CB2 in yeast (EC_{50}; 330 ± 32 nM and 240 ± 30 nM, E_{max} 54% and 74% respectively). By comparing equipotent molar ratios (EMR) in the yeast assay, relative to HU210, we calculate the selectivity for CB_1 over CB_2 of ACPA and ACEA to be 12- and 7-fold, respectively. We present similar experiments using a panel of endocannabinoids and synthetic ligands to support the pharmacological authenticity of the yeast system for functional assay of CB_1 and CB_2.

PROSTAMIDES, ANANDAMIDE CYCLOXYGENASE-2 METABOLITES METABOLISM AND PRELIMINARY PHARMACOLOGICAL CHARACTERIZATION

Vincenzo Di Marzo[1], Luciano De Petrocellis[2], Tiziana Bisogno[1], Aniello Schiano Moriello[2], Isabel Matias[1], and David Woodward[3]
[1,2]*Endocannabinoid Research Group*, Institutes of [1]Biomolecular Chemistry; [2]Cybernetics, C.N.R., Pozzuoli (NA), Italy, [3]Allergan, Irvine, CA, USA

The prostaglandin-ethanolamides (prostamides) are produced from the action of cyclo-oxygenase-2 (COX-2) on anandamide. So far prostamide E_2, D_2 and $F_{2\alpha}$ have been described, but little is known of their metabolism and pharmacology, except that they do not activate the two cannabinoid receptors. The present study was performed to determine if prostamides exhibit similar properties to their precursor anandamide with respect to inactivation mechanisms and pharmacological activity. Anandamide is transported into cells by facilitated diffusion. It is subsequently hydrolyzed to arachidonic acid and ethanolamine by Fatty Acid Amide Hydrolase (FAAH). Thus, the affinity of prostamide for the putative anandamide transporter and FAAH was determined. Furthermore, prostamide activity at the VR1 receptor was evaluated since anandamide is a VR1 receptor agonist.

Affinity of prostamides D_2, E_2 and $F_{2\alpha}$ for the anandamide transporter and FAAH was determined by competition studies *vs.* [^{14}C]anandamide. [^{3}H]Prostamide $F_{2\alpha}$ was used for cellular uptake and degradation studies in C6 and RBL-2H3 cells and in rat brain homogenates. VR1 receptor stimulation was determined by measuring $[Ca^{2+}]_i$ in cells over-expressing the recombinant human VR1 receptor.

The prostamides D_2, E_2 and $F_{2\alpha}$ did not inhibit uptake of anandamide by RBL-2H3 cells nor did they attenuate anandamide hydrolysis by rat brain homogenates, at doses up to and including 10^{-4}M. [^{3}H]Prostamide $F_{2\alpha}$ was not appreciably taken up by C6 and RBL-2H3 cells after up to 30 min incubation, nor was it significantly hydrolysed by rat brain homogenates.
At high concentrations (5-30 10^{-6}M), prostamides stimulated the VR1 receptor according to the following potency rank order: prostamide $F_{2\alpha}$ > prostamide D_2 > prostamide E_2. The effect of prostamide $F_{2\alpha}$ was blocked by 10^{-5}M capsazepine treatment. Prostamide potency was not increased by pluronic to increase cell permeability.

In conclusion, the prostamides are not substrates for the anandamide transporter or FAAH, and seem to be rather stable to inactivation (uptake + hydrolysis) mechanisms in murine cells and tissues. They exhibit weak effects on the VR1 receptor and an EC_{50} of 10^{-5}M for prostamide $F_{2\alpha}$ indicates that this is not its primary receptor target. However, since the ligand binding site on VR1 is cytosolic (De Petrocellis, L. et al., J. Biol. Chem. 276, 12856, **2001**), and prostamide $F_{2\alpha}$ is not rapidly taken up by cells, patch-clamp studies may provide a definitive interpretation of prostamide effects on this receptor.

ASSESSING THE PRESENCE OF CB_1 AND CB_2 CANNABINOID RECEPTOR mRNA IN WILDTYPE, HETEROZYGOUS AND CANNABINOID CB_2 RECEPTOR KNOCKOUT MICE

Trisha Duncan, Taujai Williams, and Nancy E. Buckley
California State Polytechnic University-Pomona, Pomona, CA 91768, USA

It has been postulated that cannabinoids such as Δ^9-tetrahydrocannabinol, the psychoactive derivative of marijuana, are immunomodulators. These effects are thought to be mediated by two cannabinoid receptors, the peripheral (CB_2) cannabinoid receptors, and to a lesser extent by the central (CB_1) cannabinoid receptors. The CB_2 cannabinoid receptor mRNA is predominantly expressed in the immune cells, whereas CB_1 cannabinoid receptor mRNA is primarily expressed in the central nervous system, although some expression of CB_1 has been reported in the immune cells. Given that the CB_1 cannabinoid receptor is thought of as a central nervous system receptor, this would support the fact there is an intimate relationship between the central nervous system and the immune system. In mice lacking the CB_2 cannabinoid receptor, we wanted to know if there was a compensatory mechanism between CB_1 and CB_2 cannabinoid receptor mRNAs.

We assessed the presence of CB_1 and CB_2 cannabinoid receptor mRNAs in the wild-type (+/+), heterozygote (+/-), and CB_2 knockout mice tissues by semi-quantitative reverse transcribed - polymerase chain reaction (RT-PCR). CB_1 and CB_2 cannabinoid receptor-transfected cell lines were used as positive controls. Our results confirmed that CB_1 cannabinoid receptor mRNA was mainly present in the brain and testes of wild type mice. Furthermore, our results showed that CB_1 cannabinoid receptor mRNA was also present in the brain and testes of heterozygote and CB_2 cannabinoid receptor knockout mice. Our results also confirmed that CB_2 cannabinoid receptor mRNA is present in spleen and thymus of wild type mice. In addition, we found CB_2 cannabinoid receptor mRNA in the testis of wild type mice. CB_2 cannabinoid receptor mRNA was also present in the spleen and thymus of the heterozygous mice, and as expected, missing in the CB_2 cannabinoid receptor knockout mice. Thus, according to our results, the CB_1 cannabinoid receptor does not compensate for the lack of CB_2 cannabinoid receptor in the CB_2 cannabinoid receptor knockout mice. However, we cannot rule out the existence of a yet unknown third cannabinoid receptor that may compensate for the lack of CB_2 cannabinoid receptor.

FURTHER CHARACTERIZATION OF THE CANNABINOID RECEPTOR INHIBITING NORADRENALINE RELEASE IN THE GUINEA-PIG HIPPOCAMPUS

Markus Kathmann, Susanna Liedtke, Karsten Flau, Agnes Redmer, and Eberhard Schlicker
Department of Pharmacology and Toxicology, University of Bonn,
Reuterstrasse 2b, D-53113 Bonn, Germany

Guinea-pig hippocampal slices preincubated with ^{3}H-noradrenaline were superfused and the effect of cannabinoid receptor ligands and of ligands at other presynaptic receptor systems on the electrically (0.3 Hz) evoked tritium overflow was studied. The evoked overflow (representing quasi-physiological noradrenaline release) was inhibited by the cannabinoid receptor agonist WIN55,212-2; the maximum inhibitory effect amounted to 60 % and the $pIC_{30\%}$ was 6.48. The concentration-response curve of WIN55,212-2 was shifted to the right by two selective CB_1 receptor antagonists, AM251 0.032 µM and AM281 0.1 µM, yielding pA_2 values of 8.42 and 7.67, respectively. By themselves, AM251 and AM281 increased the evoked tritium overflow by 53 and 49 %, respectively. The selective CB_2 receptor antagonist SR144528 1 µM did not affect the concentration-response curve of WIN55,212-2 nor did it influence the evoked overflow by itself. For the sake of comparison, the inhibitory effect of compounds activating other systems of presynaptic receptors on the noradrenergic neurone were studied as well; concentrations producing the maximum inhibitory effect at the respective receptors were used. The extent of inhibition of the evoked tritium overflow was 94 % for U-69,593 10 µM (κ opioid receptors), 82 % for nociceptin 1 µM (ORL_1 receptors) and 27 % for R-α-methylhistamine 1 µM (histamine H_3 receptors); prostaglandin E_2 1 µM (activating prostaglandin EP_3 receptors) only tended to inhibit noradrenaline release.

These results further support the conclusion drawn from our previous paper (Schlicker *et al*., *Naunyn-Schmiedeberg´s Arch Pharmacol* **1997**, *356*, 583) and based on experiments with SR141716 that the presynaptic cannabinoid receptor on the noradrenergic neurones of the guinea-pig hippocampus belongs to the CB_1 receptor subtype and is subject to an endogenous tone. Furthermore, the study shows that the maximum inhibition of noradrenaline release via CB_1 receptors exceeds that obtained via EP_3 and H_3 receptors but is lower than that obtained via ORL_1 and κ opioid receptors.

AM251, *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazol-3-carboxamide; AM281, 1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1H-pyrazole-3-carboxamide; U-69,593, [(5α,7α,8β)-(+)-*N*-(7-[1-pyrrolidinyl]-1-oxa-spiro[4,5]dec-8-yl)benzenacetamide

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ACTIVATION OF MUSCARINIC ACETYLCHOLINE RECEPTORS ENHANCES THE RELEASE OF ENDOCANNABINOIDS IN THE HIPPOCAMPUS

Jimok Kim[1], and Bradley E. Alger[1,2]
[1]Program in Neuroscience and [2]
Department of Physiology, University of Maryland
School of Medicine, Baltimore, MD 21201, USA

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BINDING, DEGRADATION AND ACTIVITY OF STEAROYLETHANOLAMIDE IN RAT C6 GLIOMA CELLS

Mauro Maccarrone, Riccardo Pauselli, Marianna Di Rienzo, and Alessandro Finazzi-Agrò
Department of Experimental Medicine and Biochemical Sciences, University of Rome
Tor Vergata, I-00133 Rome, ITALY

Stearoylethanolamide (SEA) is present in human, rat and mouse brain in amounts comparable to those of the endocannabinoid anandamide ((M. Maccarrone et al. *J. Neurochem*. 78:339, **2001**). Yet, the biological activity of SEA has never been investigated. We synthesized cold and radiolabeled SEA, in order to investigate its binding, degradation and biological activity in rat C6 glioma cells. We report that SEA binds to a specific site distinct from known cannabinoid or vanilloid receptors, and that anandamide (AEA) and capsazepine partly (~50%) antagonized this binding. Treatment of C6 cells with SEA inhibits cellular nitric oxide synthase and does not affect adenylate cyclase, whereas treatment with CB_1 agonist 2-arachidonoylglycerol activates the former enzyme and inhibits the latter. C6 cells have also a specific SEA membrane transporter, which is inhibited by nitric oxide, and a fatty acid amide hydrolase able to cleave SEA. In these cells SEA shows pro-apoptotic activity, due to elevation of intracellular calcium, activation of the arachidonate cascade, and mitochondrial uncoupling. Nitric oxide further enhances SEA-induced apoptosis. Moreover, the CB_1 cannabinoid receptor-mediated decrease of cAMP induced by AEA in C6 cells is potentiated by SEA, suggesting that this compound has also an "entourage" effect. Taken together, this study shows that SEA is an endocannabinoid-like compound which binds to and is transported by new components of the endocannabinoid system. It seems noteworthy that degradation and pro-apoptotic activity of SEA are regulated by nitric oxide in the opposite way compared to that reported for AEA.

ARE CB_1 AND CB_2 RECEPTOR PROTEINS DIFFERENTLY EXPRESSED IN WILD TYPE, CB_2 CANNABINOID RECEPTOR KNOCKOUT, AND HETEROZYGOUS MICE

K.Martinez, and N.E. Buckley
California State Polytechnic University-Pomona, Pomona, CA 91768

Cannabinoid receptors CB_1 and CB_2 are expressed mainly in neuronal and immune cells, respectively. These receptors belong to the superfamily of transmembrane G protein coupled receptors. In this study we attempt to determine whether CB_2 cannabinoid knockout mice will express CB_2 or CB_1 cannabinoid receptors differently than heterozygote and wild type mice. The importance of this study is that it will give us an insight into the relevance of the presence of cannabinoid receptor proteins in diverse tissues. If cannabinoid receptors are key in the function of a specific tissue, we want to determine whether CB_1 cannabinoid receptors will compensate for the lack of the CB_2 cannabinoid receptor in tissues (i.e. spleen, thymus) from the CB_2 cannabinoid receptor knockout mice.

Diverse tissues were dissected from wild type, knockout, and heterozygous mice. Proteins from these tissues were assayed for the presence of CB_1 and CB_2 receptors using SDS-PAGE, western blotting and immunoblotting techniques using antibodies to CB_1 and CB_2 receptor proteins. The protein expression we obtain from the wild type, knockout, and heterozygous mice tissues can be compared to determine if these receptors have a compensatory mechanism. In addition, immunocompromised wild type mice will be analyzed by the same methods to determine if the cannabinoid receptor protein expression pattern is different from competent mice. This will give us an insight into the role the cannabanoid system may play in the immune response.

PRESENCE AND REGULATION OF THE ENDOCANNABINOID SYSTEM IN HUMAN DENDRITIC CELLS

Isabel Matias[1], Pierangelo Orlando[2], Joel Pestel[3],
Michel Salzet[4], and Vincenzo Di Marzo[1]
[1,2]Endocannabinoid Research Group, Institutes of [1]Biomolecular Chemistry and
[2]Cybernetics, C.N.R., Pozzuoli (NA), Italy; [3]Inflammatory Reaction and
Allergic Diseases Department, INSERM Unit 416, Pasteur Institute, Lille, France;
[4]Laboratoire de Neuroimmunité des Annélides, Université des
Sciences et Technologies de Lille, Villeneuve d'Ascq, France

Cannabinoid receptors and their endogenous ligands, the endocannabinoids, have been detected in several blood immune cells, including monocytes/macrophages, basophils and lymphocytes. However, dendritic cells, which play a key role in the pathology of allergy, autoimmunity and cancer, have never been investigated. Here we have analyzed human dendritic cells for the presence of the endocannabinoids, anandamide and 2-arachidonoylglycerol (2-AG), the cannabinoid CB_1 and CB_2 receptors, and one of the enzymes mostly responsible for endocannabinoid hydrolysis, the fatty acid amide hydrolase (FAAH).

By using a very sensitive liquid chromatography-atmospheric pressure chemical ionization-mass spectrometric (LC-APCI-MS) method, lipids extracted from immature dendritic cells were shown to contain 2-AG, anandamide and the anti-inflammatory anandamide congener, *N*-palmitoylethanolamine (PEA) (2.1 ± 1.0, 0.14 ± 0.02 and 8.2 ± 3.9 pmol/10^7 cells, respectively). The amounts of 2-AG, but not anandamide or PEA, were significantly increased on maturation of cells induced by lipopolysaccharide (LPS) or the mite allergen DERP1 (2.8 and 1.9-fold, respectively). By using both RT-PCR and Western immunoblotting, dendritic cells were also found to express measurable amounts of CB_1 and CB_2 receptors and of FAAH. Cell maturation did not consistently modify the expression of these proteins, although in some cell preparations a decrease of the levels of both CB_1 and CB_2 mRNA transcripts was observed after LPS stimulation.

These findings demonstrate for the first time that the endogenous cannabinoid system is present in human dendritic cells and can be regulated on activation of these cells.

AUTORADIOGRAPHIC STUDY OF CANNABINOID RECEPTOR DISTRIBUTION AND FUNCTIONALITY IN THE DEVELOPING HUMAN BRAIN

S. Mato, and A. Pazos
Departments of Physiology and Pharmacology,
University of Cantabria, Santander 39011, Cantabria, Spain

Several lines of evidence suggest that the endogenous cannabinoid system is present and functionally active during brain development (Berrendero et al., Development, 125, 3179, **1998**; Fernández-Ruiz et al., Life Sci., 65, 726, **1999**). In the present study, we have investigated the pre- and postnatal distribution and G protein coupling ability of cannabinoid receptors in *postmortem* human brain samples corresponding to different stages of development (fetal, neonatal and infant), aging from 19 weeks of gestation to 8 years of life. The cannabinoid receptor protein was quantified by autoradiographic techniques using [^{3}H]CP55,940 as a ligand. The functional coupling to G proteins was studied by WIN55,212-2-stimulated [^{35}S]GTPγS autoradiography. The specifity of WIN55,212-induced stimulation was assessed in consecutive sections coincubated with the cannabinoid antagonist SR141716A.

In the fetal brain, [^{3}H]CP55,940 specific binding was observed as early as 19 weeks of gestation. The density and anatomical distribution of cannabinoid receptors in the developing human brain was, in general terms, comparable to that found in the adult. In fact, very high levels of cannabinoid receptor binding sites (> 35 fmol/mg t.e.) were observed in the striatum and hippocampal formation, whereas high concentrations of receptor protein (20-35 fmol/mg t.e.) were measured in cortical regions and cerebellum. Interestingly, very high densities of cannabinoid receptors were detected in certain white matter areas, such as the pyramidal tract, wich are practically devoid of these receptors in the adult human brain. This atypical localization was only observed in the fetal brain from the earliest gestational period, and not in the neonatal and infant brains.

On the other hand, the cannabinoid agonist WIN55,212-2 stimulated [^{35}S]GTPγS binding in fetal human brain from the earliest gestational age with a similar intensity to that observed in the adult cases. The anatomical distribution of WIN55,212-2-induced G protein activation in the fetal, neonatal and infant brains was fully comparable to the pattern of cannabinoid receptor protein localization in these cases, including the transient hyperexpression in the pyramidal tract.

These results suggest that cannabinoid receptors emerge and are functionaly active since relative early stages of human brain development. This temporal pattern of appearance, along with the transient and atypical hyperexpression of cannabinoid receptors in fiber-enriched areas, accounts for an specific role of the endogenous cannabinoid system in the regulation of brain development in the human being.

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CANNABINOID-INDUCED FOS EXPRESSION WITHIN A10 DOPAMINERGIC (DAergic) NEURONS: SUBREGION-SPECIFIC MEDIATION BY α_1-ADRENERGIC RECEPTORS

Sachin Patel, and Cecilia J. Hillard
Department of Pharmacology and Toxicology
Medical College of Wisconsin, Milwaukee, WI 53226

It is known that cannabinoids increases mesocorticolimbic dopaminergic neurotransmission. The purpose of this study was to determine the effects of cannabinoid agonists on the pattern of Fos protein expression within A10 DAergic neurons of the ventral tegmental area (VTA), and to determine whether this expression is mediated by α_1-adrenergic receptors. We utilized double-labeled immunohistochemistry, using Fos as a marker of neuronal activation and tyrosine hydroxylase (TH) to identify DAergic neurons. CP55940 and WIN55212-2, but not its inactive isomer WIN55212-3, induced Fos expression within the VTA and locus coeruleus. The CB_1 receptor agonist CP55940 (0.1-10 mg/kg i.p.) increased the total number of Fos-like immunoreactive (Fos-li) cells within the parabrachial (PBP) and caudal linear (CL) subdivisions of the VTA and the number of Fos-li DAergic cells within the PBP, paranigral (PN), CL, and interfascicular (IF). Furthermore, CP55940 increased the proportion of Fos-li DAergic cells with respect to the total number of Fos-li cells in each subdivision in the PBP, IF, and CL. Pretreatment with the α_1-adrenergic receptor antagonist prazosin (1 mg/kg i.p.) reduced the total number of CP55940-induced Fos-li cells within the PBP and the CL; the number of CP55940-induced Fos-li DAergic neurons in the PBP, PN, and IF, and the proportion of Fos-li DAergic neurons within the PBP and PN. These data suggest that cannabinoid-induced increases in forebrain DA release are dependent, in part, upon somatodendritic excitation of DAergic neurons by noradrenergic afferents. These data also suggest a functional heterogeneity within A10 DAergic neurons based not only on topography but differential mediation by α_1-adrenergic receptors of the response to cannabinoids.

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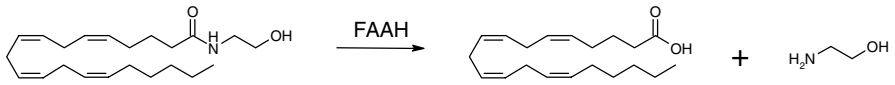
INHIBITION OF FATTY ACID AMIDE HYDROLASE BY METHYL ARACHIDONYLFLUOROPHOSPHONATE (MAFP) IN BOVINE SERUM ALBUMIN (BSA)

Susanna M. Saario, Risto O. Juvonen[1], Tomi Järvinen, and Jukka Gynther
Department of Pharmaceutical Chemistry, and [1]Department of Pharmacology and Toxicology, University of Kuopio, P.O. Box 1627, FIN-70211 Kuopio, Finland

Fatty acid amide hydrolase (FAAH) is a membrane associated enzyme that catalyzes the hydrolysis of the endocannabinoid arachidonyl ethanolamide (AEA) to lipid-soluble arachidonic acid and water soluble ethanolamine, as shown below:



In several studies, BSA has been used for the *in vitro* assay of FAAH activity. In the present study, an end-point enzymatic assay was developed to quantify FAAH activity with tritium labelled arachidonyl ethanolamide [ethanolamine 1-^{3}H]. In addition, the effect of BSA on FAAH activity and its inhibition was also studied, because BSA may change the free concentration of AEA and inhibitors during incubation. Male Han/Wistar rat brain homogenate was used as a source of FAAH. Ethyl acetate appeared to be an optimal solvent for extracting arachidonic acid from aqueous phase. Incubation time did not exceed 10 min, and the amount of brain homogenate was typically 5 – 100 µg/sample. The reactions followed Michaelis-Menten kinetics, and BSA decreased the K_m-value of AEA (Table 1) with no significant change in $V_{max.}$

Table 1. Michaelis-Menten parameters for FAAH-catalyzed hydrolysis of AEA (95 % confidence intervals in parentheses).

	Absence of BSA	**Presence of BSA (0.25 %)**
K_m (µM)	7.5 (6.1-9.0)	3.0 (2.2 -3.9)
V_{max} (µmol min^{-1} mg^{-1} prot.)	12.5 (11.6-13.4)	12.9 (11.9 -13.8)

Methyl arachidonylfluorophosphonate (MAFP) is a well-known irreversible inhibitor of FAAH (Deutsch et al., Biochem. Pharmacol., **1997**, 53, 257). Its IC_{50}-value was 0.3 nM when preincubated with brain homogenate, however, its IC_{50}-value was 2 nM when MAFP and AEA were added simultaneously. Interestingly, 10 nM MAFP did not inhibit FAAH when 0.5 % BSA was used in the incubation mixture. This is probably due to the fact that BSA prevents the interaction between FAAH and MAFP.

In conclusion BSA has a significant effect on reactions catalyzed by FAAH, which may due to binding properties of AEA and MAFP to BSA.

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PURIFICATION AND CHARACTERIZATION OF CB_2 CANNABINOID RECEPTOR EXPRESSED IN *PICHIA PASTORIS*

Z. H. Song, Jian Cai, William Pierce, and Wenke Feng
Department of Pharmacology and Toxicology, University of Louisville
School of Medicine, Louisville, KY 40292, USA

Methylotrophic yeast *Pichia pastoris* has been developed as an excellent host for the large-scale expression of proteins. Similar to bacterial expression system, the growing of yeast is fast and inexpensive. In addition, yeast cells are eukaryotes, and therefore they have the machinery for post-translational modifications. To facilitate the investigation of structure and function relationships of CB_2, in current study this receptor is expressed in *P. pastoris*.

The expression plasmid was constructed in which the CB_2 gene is under the control of the highly inducible promoter of *P. pastoris* alcohol oxidase 1 gene. A c-myc epitope and a polyhistidine tag were introduced at the C-terminal of the CB_2 to permit immunological detection and purification. The expression plasmids were transformed into yeast cells by electroporation, and recombinant yeast cells were selected by growing on Zeocin-containing plates. Subsequently, the expressed CB_2 was characterized by Western blot analysis and ligand binding assays. Furthermore, the receptor was purified by metal chelating chromatography, and the purified CB_2 was characterized by mass spectrometry.

In the membrane preparations of CB_2 gene transformed yeast cells, Western blot analysis detected the expression of CB_2 protein. Radioligand binding assays demonstrated that the CB_2 expressed in *P. pastoris* has a pharmacological profile similar to that of the receptor expressed in mammalian systems. Following purification by metal chelating chromatography, the purified CB_2 preparations were subjected to digestion by trypsin. MALDI-TOF and ESI-IT mass spectrometry analysis of the peptides extracted from tryptic digestions confirmed the identity of the purified CB_2.

In conclusion, these data demonstrated for the first time that epitope-tagged CB_2 cannabinoid receptor can be expressed in *P. pastoris* for convenient purification and structural characterization by mass spectrometry.

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ACTIVATION OF *N*-ACYLETHANOLAMINE-RELEASING PHOSPHOLIPASE D BY POLYAMINES

Kazuhito Tsuboi[1], Qian Liu[1], Takeharu Tonai[2], and Natsuo Ueda[1]
[1]Department of Biochemistry, Kagawa Medical University, Miki, Kagawa 761-0793, Japan,
[2]Department of Orthopedic Surgery and Clinical Research Institute,
National Zentsuji Hospital, Kagawa 765-0001, Japan

Anandamide and other long-chain *N*-acylethanolamines are biosynthesized from their corresponding *N*-acyl-phosphatidylethanolamines by the catalysis of a phophodiesterase of the phospholipase D type (Schmid, Chem. Phys. Lipids **2000**, *108*, 71; Hansen et al., Chem. Phys. Lipids **2000**, *108*, 135). Recently, we solubilized this enzyme from the particulate fraction of rat heart with the aid of 1% (w/v) octylglucoside, and partially purified the solubilized enzyme. With this partially purified enzyme, we found potent activation of the enzyme by millimolar order of Ca^{2+} (Ueda et al., Biochim. Biophys. Acta **2001**, *1532*, 121). Here, we show that the enzyme is also potently stimulated by some polyamines. The partially purified enzyme was allowed to react with 100 μM *N*-[^{14}C]palmitoyl-phosphatidylethanolamine in the presence of increasing concentrations of three polyamines (spermine, spermidine, and putrescine). The produced *N*-[^{14}C]palmitoylethanolamine was then separated by thin-layer chromatography and was quantified by a bioimaging analyzer BAS1500. The specific enzyme activity was dose-dependently increased by polyamines, but not by ethanolamine (a monoamine). Amongst the polyamines tested, spermine was the most potent with an EC_{50} value of about 0.1 mM, which is within the physiological range. Spermine at 10 mM increased the specific activity 27-fold to 53 nmol/min/mg protein at 37°C. However, no synergistic effect of spermine and Ca^{2+} was observed, suggesting that these two substances activate the enzyme by the same mechanism. The partially purified enzyme was also active with *N*-arachidonoyl-phosphatidylethanolamine to release anandamide in the presence of spermine. These results suggest that polyamines are endogenous activators to regulate the biosynthesis of anandamide and other long-chain *N*-acylethanolamines.

EFFECTS OF THE ANANDAMIDE UPTAKE BLOCKER ON MORPHINE SELF-ADMINISTRATION IN MALE WISTAR RATS

J.M. Trigo, J.A. López-Moreno, F. Rodríguez de Fonseca, and M. Navarro
Laboratorio de Psicobiología, Facultad de Psicología, Universidad Complutense, Campus de Somosaguas, Madrid

Previous studies developed in our laboratory showed that the cannabinoid CB_1 receptor antagonist SR141716A was able of modify heroin and morphine self administration in both mice and rats using continuous reinforcement paradigm (Navarro et al, J. Neuroscience, 21(**2001**):5344). The present study was oriented to study whether the anandamide uptake blocker AM404 (0.4, 2, 10 y 50 mg/kg, i.p.) was capable of modify intravenuous morphine self-administration in male Wistar rats, using both continuous reinforcement (FR 1) or progressive ratio paradigms. The results obtained suggest that AM404 is not able of modifying morphine self-administration at doses that target the anandamide transporter. However, the highest dose used (50 mg/kg) decreased operant responses for morphine. Since, as described, AM404 loses its selectivity for the transporter at micromolar concentrations, the effects of the 50 mg/kg dose of AM404 might not be dependent on the blockade of anandamide transporter, but on its effects on either vanilloid or adrenergic receptors (De Petrocellis et al., FEBS lett 483 (**2000**): 52; Beltramo et al., J. Neuroscience 20(**2000**): 340). These results sugest that morphine self-administration apparently does not to affect anandamide production in the reward circuit.

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LIGAND FISHING WITH ANANDAMIDE BY MASS SPECTROMETRY : POLYMYXIN BITES, BUT NOT ANALOGUES OF *N*-PALMITOYLETHANOLAMINE

Séverine Vandevoorde[1], Raoul Rozenberg[2], Jean-Louis Jiwan Habib[2], Christopher J. Fowler[3], and Didier M. Lambert[1]
[1]Catholic University of Louvain, Medicinal Chemistry and Radiopharmacy unit, 1200 Brussels, Belgium; [2]Catholic University of Louvain, Mass spectrometry laboratory, 1348 Louvain-la-Neuve, Belgium; [3]Departments of pharmacology and Clinical Neuroscience, Umeå University, Sweden

Electrospray ionisation mass spectrometry (ESI-MS) is a « soft » ionisation method which allows analysis of even weakly-bound non-covalent complexes. If we consider the complex association A + B $\rightarrow$ AB, then, when a solution containing a mixture of A and B is introduced into a mass spectrometer, the resulting spectrum may show ion signals not only due to A and B, but also to AB. This method for the detection of non-covalent interactions has been named « ligand fishing » approach.

Wang et *al.*[1] discovered that polymyxin B (PMB) binds to anandamide (AEA) and consequently, inhibits its cytotoxic effect. The authors used PMB-immobilized beads column and observed retention of AEA on the beads, and elution only when ethanol was used.

Using ESI-MS to confirm the non-covalent binding between AEA and PMB, we first injected in the spectrometer equimolar solutions of AEA and PMB in methanol/water as solvent, but we did not observed the formation of complex in these conditions. The formation of complex by MS/MS has been confirmed in contrary, with saline and ethanol as solvents.

Because the interaction between AEA and PMB is probably driven primarily by hydrophobic forces, the analogues of *N*-palmitoylethanolamine (PEA) known as good inhibitors of AEA metabolism (*N*-palmitoylethylamine, *N*-palmitoylisopropylamine, *N*-oleylethanolamine, *N*-oleylethylamine and *N*-oleylisopropylamine)[2] might form a AEA-PEA complex. In this case, the inhibition of the anandamide metabolism might be due to the prevention of AEA to access to its degradative sites instead of a direct action at the uptake and/or fatty acid amide hydrolase level. ESI-MS has been used to (in)validate this hypothesis. We were only able to detect complex formation at high concentration (1 mM), amply higher than the concentration required (10 µM) for inhibition of AEA metabolism. We can thus confirm that inhibition of AEA metabolism by analogues of PEA is not caused by interaction between the two compounds.

[1] Wang et *al.*, *FEBS Letters*, **470**, 151-155 (2000)
[2] K.O. Jonsson, S. Vandevoorde, D.M. Lambert, G. Tiger and C. Fowler, *Br. J. Pharmacol.*, **133**, 1263-1275 (2001)

MODULATION OF THE ARACHIDONIC ACID CASCADE: PATHWAY TO ANANDAMIDE'S EFFECTS?

Jenny L. Wiley, and Billy R. Martin
Department of Pharmacology and Toxicology,
Virginia Commonwealth University, Richmond, VA 23298-0613, USA

One of the major metabolic pathways for degradation of anandamide is the arachidonic acid cascade. Anandamide is metabolized to arachidonic acid and polar metabolites through action of fatty acid amide hydrolase (FAAH; Deutsch & Chin, **1993**, Biochem Pharmacol, 46, 791-796). Arachidonic acid may be further metabolized via cyclooxygenase (COX) and lipoxygenase (LOX) enzymes to thromboxane A_2, prostaglandins, and leukotrienes. The purpose of the present study was to investigate the pharmacological effects of modulation of the arachidonic acid cascade. Mice were tested in the cannabinoid tetrad tests for suppression of locomotor activity, antinociception in the tail flick test, decreased rectal temperature, and catalepsy. As reported last year at this meeting, anandamide produced all four of these effects whereas arachidonic acid suppressed spontaneous activity and had antinociceptive effects, but did not affect the other two measures. SR141716A did not block the pharmacological effects of either drug. In contrast, in the present study we found that the reversible COX inhibitor ibuprofen (20 mg/kg) reversed the *in vivo* effects of arachidonic acid, but neither 20 nor 40 mg/kg ibuprofen altered anandamide's effects. Tests with other blockers of the arachidonic acid pathway confirm this observation that anandamide's effects are not mediated by conversion to arachidonic acid. Nimesulide (a selective COX-2 inhibitor), esculetin (an inhibitor of 5-LOX and 12-LOX), and phenidone (a dual COX/LOX inhibitor) did not alter the pharmacological effects of anandamide nor did they produce cannabimimetic effects of their own. The steroidal anti-inflammatory and phospholipase blocker, dexamethasone, also did not reverse anandamide's effects. These results suggest that the failure of SR141716A to block the *in vivo* effects of anandamide (administered alone without PMSF) is not due to anandamide's rapid metabolism to arachidonic acid. Further, they suggest that non-CB_1, non-CB_2 receptors and/or pathways other than the arachidonic acid cascade may play a role in anandamide's effects.

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CANNABINOIDS PRODUCE MNEMONIC DEFICITS THROUGH A $GABA_A$ SENSITIVE SYSTEM

Stephen A. Varvel, Aron H. Lichtman, and Emmanuel Anum
Department of Pharmacology and Toxicology, PO Box 980613,
Richmond, VA 23233-0613, USA

Delta-9-THC and other cannabinoids have long been known to produce deficits in certain aspects of learning and memory, widely believed to be due in large part to activation of CB_1 receptors located in the hippocampus. Since these receptors are predominantly localized presynaptically on GABAergic nerve terminals, it has been hypothesized that cannabinoids may exert their effects in the hippocampus by altering GABAergic transmission. Furthermore, recent evidence suggests that hippocampal GABAergic transmission may be tonically influenced by the endocannabinoid system. In the present study C57Bl/6 mice were trained to perform a working memory version of the Morris water maze, in which they were required to learn the location of a hidden platform which was placed randomly in one of 24 locations prior to each session. As has been previously demonstrated, THC produced dose-related deficits in this task at relatively low doses, which were blocked by coadministration of the CB_1 antagonist SR141716A. We now report that the deficits induced by a maximally-effective dose of THC (10 mg/kg) were reversed by coadministration of 1 mg/kg of the GABA-A antagonist bicuculline ($t[27] = 2.5$, $p < 0.05$). Conversely, 30 mg/kg CGP 36742, a GABA-B antagonist, had no effect on the THC-induced deficits ($t[19] = 1.2$, $p = 0.24$), though this dose did antagonize the deficits produced by the $GABA_B$ agonist baclofen ($t[13] = 2.3$, $p < 0.05$). In a subsequent set of experiments, the effects of muscimol and baclofen were evaluated in CB_1 knockout mice (CB_1 -/-) and corresponding wildtypes (CB_1 +/+) in order to assess whether the endocannabinoid system may exert a tonic influence on GABAergic transmission. Interestingly, CB_1 -/- mice were more sensitive to the disrupting effects of muscimol than were CB_1 +/+ mice. A similar effect was not observed for baclofen, as both groups were disrupted at similar doses. These results suggest that mediation of THC-induced working memory deficits may involve increases in activity at GABA-A, but not GABA-B receptors. Furthermore, CB_1 -/- mice displayed increased sensitivity to a GABA-A receptor agonist, suggesting this system may be tonically influenced by endocannabinoids.

FUNCTIONAL CONSEQUENCES OF SYNAPTIC MODULATION BY CANNABINOIDS IN THE RODENT NUCLEUS ACCUMBENS

Gregory L. Gerdeman, and Carl R. Lupica
Department of Pharmacology, University of Arizona Health Sciences Center, Tucson, Arizona 85724, USA

The nucleus accumbens (NAc) is a brain structure that receives and integrates synaptic inputs from a variety of cortical and limbic system areas. As a key component of the mesolimbic "reward" circuitry, the NAc is believed to play important roles in the generation of motivational states and mediating the reinforcing properties of commonly abused drugs. Recent studies have demonstrated that CB_1 receptor activation inhibits neurotransmission at both excitatory and inhibitory synapses in the NAc[1,2]. To better understand how these opposing modulatory actions ultimately influence the activity and function of NAc medium spiny neurons (and thus NAc output), we have begun testing effects of the CB_1 agonist WIN55,212-2 on locally evoked postsynaptic potentials (PSPs) in these neurons using whole-cell recordings in the current clamp mode. Preliminary data suggest that cannabinoid effects on NAc neurons may be rather complex, depending on the resting membrane potential of the neuron and the strength of the synaptic input. As an example, the evoked PSPs presented in figure 1 show that at a constant stimulus intensity of 1.1 mA (*left panel*), application of 1 μM WIN led to a decrease in peak PSP amplitude, consistent with inhibition of excitatory afferents. However, in the same neuron, the response to a stimulus amplitude of 2.0 mA was not similarly reduced by WIN, and instead a previously unseen compound PSP was evoked, leading to a longer lasting depolarization (*right panel*). This suggests that cannabinoids may act to increase NAc output under situations of strong afferent input, perhaps by reducing GABAergic inhibition.

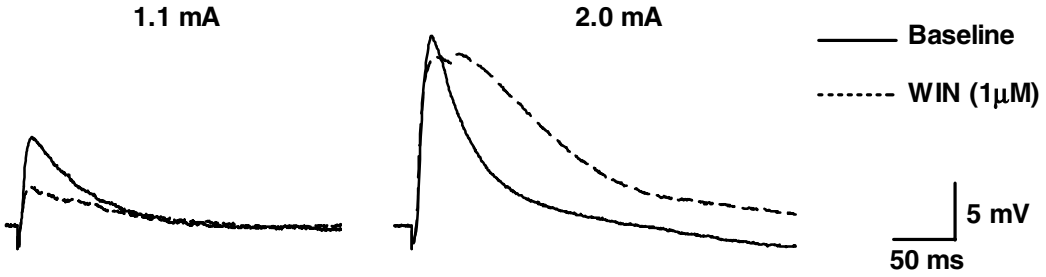

Figure 1. Actions of WIN55,212-2 on evoked PSPs in a NAc medium spiny neuron.

Further experiments will utilize pharmacological and physiological approaches to elucidate whether excitatory or inhibitory synaptic inputs are modulated by the same or different degrees by cannabinoids in the NAc.

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2. Robbe D, *et. al., J. Neurosci.* **2001** Jan 1;21(1):109-16.

CANNABINOID-INDUCED RECIPROCAL ACTIVITY IN TWO NEURONAL POPULATIONS OF THE VENTRAL TEGMENTAL AREA

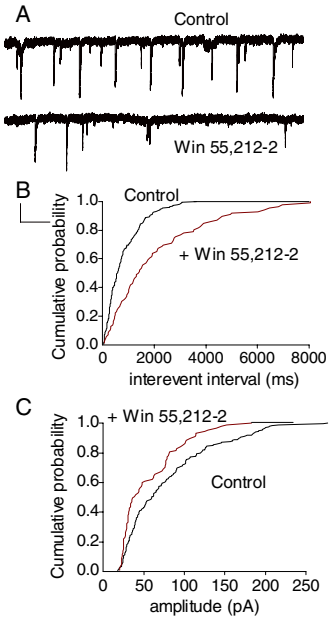
Seiji Hayashizaki, Joseph Cheer, and Sam Deadwyler
Department of Physiology and Pharmacology, Wake Forest University,
Winston-Salem, North Carolina 27157, USA

Central cannabinoid receptors (CB_1) have been found to mediate the effects of cannabinoids in the mesolimbic dopamine (DA) system using a variety of techniques (see Ameri et al., **1999** for review). The aim of the present experiments was to determine the role of cannabinoids in the activity of cells from the ventral tegmental area (VTA). In the first experiment, *in vivo* recordings were carried out in chloral hydrate anesthetized animals. The potent cannabinoid agonist HU210 was administered (i.p.) to rats implanted with multiple single-electrode arrays in the VTA. In this preparation, HU210 given at a dose that produces catalepsy unmasked three populations of cells. Thirty out of 47 cells showed a mean pre-HU210 firing rate increase of ~ 68 % (4.2 ± 1 to 13.2 ± 3.5 Hz, $F_{(2,121)} = 3.14$; $p<0.05$). These units also met criteria for dopaminergic cells and were inhibited by an apomorphine challenge (ED_{50} ~ 40 $\mu g.kg^{-1}$). Another population of cells exhibited a ~ 49 % decrease in firing rate following HUI210 injection (9/47 neurons; 8.3 ± 1.7 to 5.6 ± 2.3 Hz, $F_{(2,121)} = 3.14$; $p<0.05$) these cells also fitted non-DA cell criteria and were insensitive to apomorphine. Eight out of 47 cells failed to show a change in firing rate of more than 20 % (3.5 ± 1.2 to 4.3 ± 1.0 Hz). The second experiment was aimed at examining the effects of another high-efficacy cannabinoid agonist, WIN55,212-2 (WIN) delivered intravenously, to freely moving rats previously trained to self-administer cocaine. Under these conditions the three populations of cells observed in anesthetized rats were still present and the reciprocity in firing caused by the cannabinoid injection was more pronounced. Cells that were excited by WIN exhibited a 291 % increase in firing rate compared to pre-WIN (n=5, 1.22 ± 0.1 to 4.7 ± 1.6 Hz, $F_{(2,100)} = 9.16$; $p < 0.05$) and were inhibted by cocaine in the self-administration paradigm. WIN inhibited the firing rate of the majority of cells (n=17) by 98 % compared to pre-WIN (1.01 ± 0.08 to 0.23 ± 0.03 Hz, $F_{(2,277)} = 29.88$; $p<0.05$). The latter population of cells was not ihnibited by cocaine self-administration. Six neurons remained unaffected by the WIN treatment. Changes in firing were dose-dependent and plateaued with the onset of catalepsy and returned to pre-injection levels following recovery. Use of the CB_1 receptor antagonist SR141716A (SR) effectively antagonized the changes in firing rate in both experiments. In anesthetized rats, baseline firing rate (4.6 ± 1.2 Hz) was not significantly altered when HU210 was given 15 min following an SR injection (3.9 ± 0.9 Hz; $F_{(2,129)} = 3.91$, $p<0.05$). In awake behaving rats, SR reversed the excitation caused by WIN; post SR firing rate did not differ significantly from pre-WIN (0.64 ± 0.05 Hz and 0.93 ± 0.06 Hz, pre-WIN and post-SR, respectively). SR also reversed the effect of WIN administration on cells inhibited by WIN to pre-WIN firing rate (0.82 ± 0.03 Hz and 0.77 ± 0.03 Hz, pre-WIN and post-SR, respectively). When given on its own, SR had no effects in the firing rate of simultaneously recorded units in the anesthetized animal and showed no intrinsic effects on behavior or on cell activity when injected to awake behaving rats. These results suggest that cannabinoid agonists elicit differential responses of VTA neurons through CB_1 receptors in anesthetized or freely moving rats. We have demonstrated, for the first time, that cannabinoids are also capable of inhibiting cells in the ven tral tegmentum.

MODULATION OF CONSTITUTIVE GABAERGIC NEUROTRANSMISSION IN HIPPOCAMPAL INTERNEURONS BY CANNABINOIDS

Arthur C. Riegel[1], Alexander F. Hoffman[2], and Carl R. Lupica[1]
[1]Department of Pharmacology, University of Arizona Health Sciences Center, Tucson, AZ, 85724, and [2]Cellular Neurobiology Branch, National Institute on Drug Abuse, Intramural Research Program, National Institutes of Health, Baltimore, Maryland 21224

Both experimental and anecdotal clinical evidence suggest that cannabinoids (CBs) may disrupt the processes underlying the formation of memories. One brain region commonly associated with memory encoding and also highly enriched with CB_1 receptors is the hippocampus. Previous work in our laboratory indicates that by inhibiting voltage-dependent calcium channels, CB_1 receptor activation inhibits the release of GABA (*Hoffman and Lupica, J.Neurosci, 20:* ***2000***) onto CA1 pyramidal neurons, the principal output cells of the hippocampus. The source of this GABA is a population of interneurons whose activity is controlled in part by innervation by other GABAergic interneurons. Here, we test the hypothesis that the output of CA1 pyramidal neurons can be modulated indirectly through CB modulation of synaptic input to interneurons. These studies used whole-cell voltage clamp recordings from CA1 interneurons identified with DIC microscopy in rat hippocampal slices, and examined the effects of CB_1 receptor activation on inhibitory synaptic inputs to these cells.



Electrophysiological recordings were obtained from GABAergic interneurons with somata residing in hippocampal stratum radiatum (*s.r.*). Spontaneous inhibitory postsynaptic currents (sIPSCs) were isolated using blockers of ionotropic glutamate (APV, 40µM; DNQX, 10µM) currents. In 60% of the interneurons examined (n=20), the frequency of sIPSCs declined (e.g., increased inter-event interval) (-38.2% ± 7.3%) during superfusion of the CB agonist WIN55,212-2 (1 µM) (Figs. A & B, $p<0.001$ K-S test, *left*). Synaptic events possessing large amplitude, fast decaying kinetics appeared particularly susceptible to WIN, which often evoked a statistically significant decline in sIPSC amplitude (Fig C, $p<0.001$ K-S test, *left*). In the remaining neurons, the sIPSC frequency in the presence of WIN remained either unchanged (+3.3% ± 2.2%, n=5) or in one instance increased (+33.0%). In many neurons, the WIN-evoked decrease in frequency reversed upon superfusion of the CB_1 antagonist SR141716A (1µM), which alone had no effect on the sIPSCs. These data suggest that in at least one population of *s.r.* GABAergic interneurons, the activation of CB_1 receptors reduces spontaneous, action potential-dependent IPSCs and thus, ultimately contributes to the control of CA1 pyramidal neuron output.

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INVESTIGATING CB_2 RECEPTOR-MEDIATED IMMUNOMODULATION OF T-CELL PROLIFERATION AND TH1/TH2 CYTOKINE PRODUCTION

Diep N. Burbridge, and Nancy E. Buckley
Department of Biological Sciences
California State Polytechnic University-Pomona ,Pomona, CA, 91768 USA

The discovery of two cannabinoid receptors in the early 1990s has enhanced our understanding of how cannabinoid compounds such as Δ^9-tetrahydrocannabinol (THC) affect human physiology. Because the central cannabinoid (CB_1) receptor is predominantly found in the central nervous system and the peripheral cannabinoid (CB_2) receptor is highly expressed in immune cells, it has been postulated that the CB_1 receptor mediates cannabinoids' psychoactive effects and the CB_2 receptor mediates their immunomodulatory effects. Some of the immunomodulatory effects under investigation in this study involve changes in T-cell proliferation, as well as modulation of T helper cell (Th) cytokine production. It has been reported that cannabinoids inhibit T-helper 1 (Th1) cell-mediated immunity and enhance T-helper 2 (Th2) humoral immunity, suggesting that cannabinoids can induce humoral immunity over cell-mediated immunity. As a result, the shift in the Th1/Th2 balance may decrease immunity to diseases that rely on cell-mediated immunity to provide resistance to infection.

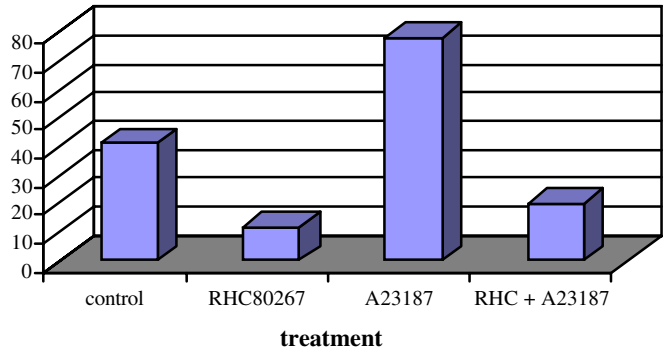
In this study, I tested the hypothesis that the CB_2 receptor mediates a cannabinoid-induced change in T-cell proliferation and a shift in the Th1/Th2 balance. T-cell proliferation, as well as the production of two Th1 cytokines (IL-2 and IFN-γ) and two Th2 cytokines (IL-4 and IL-10) were measured in primary splenocyte cultures of two groups of mice: the CB_2 cannabinoid receptor knockout mouse, a mouse deficient in the CB_2 cannabinoid receptor gene, and wild-type mice. The cells were treated with the T-cell activator Concanavalin A (2.5 μg/ml, 5 μg/ml) and the cannabinoid WIN55,212 (0.01 μM, 0.1 μM, 1 μM). Seventy-two hours post-culture, a BrdU assay was performed to measure T-cell proliferation. ELISA cytokine assays were subsequently performed to measure IL-2, IFN-γ, IL-4, and IL-10. Preliminary data show similar proliferation activity in Concanavalin-A-treated cells of both wild-type and knockout cultures, independent of cannabinoid treatment. Early cytokine results show a decrease in IL-4 and IL-10 secretions (Th2 cytokines) in both wild-type and knockout cultures for all concentrations of WIN55,212. Th1 cytokine data indicate that IFN-γ secretions are inhibited by WIN55,212 in wild-type cultures but stimulated in knockout cells. These findings suggest that the CB_2 receptor may play a role in IFN-γ secretions but not in IL-4 and IL-10 production.

CULTURED RAT MICROGLIAL CELLS PRODUCE 2-ARACHIDONYL GLYCEROL AND ANANDAMIDE, AND EXPRESS THE CB_2 RECEPTOR

Erica J. Carrier, Christopher S. Kearn, Maria Delgado, Andrew Barkmeier,
Nicole Breese, David Klick, Sandra L. Pfister, Kasem Nithipatikom,
William B. Campbell, and Cecilia J. Hillard
Department of Pharmacology, Medical College of Wisconsin, Milwaukee, WI 53226

Microglial cells, as the phagocytes and antigen-presenting cells of the central nervous system, are activated in such disease processes as ischemic stroke and multiple sclerosis. Because it has been shown that macrophages are capable of producing endocannabinoids, we have examined endocannabinoid production in a colony stimulating factor (CSF)-dependent cultured rat microglial cell line using reverse phase high pressure liquid chromatography (HPLC) and liquid chromatography-mass spectroscopy (LC-MS). We determined that cultured microglial cells robustly produce the endocannabinoids 2-arachidonyl glycerol (2-AG), at a yield of ~70000 pg/sample, as well as anandamide in much smaller quantities (~250 pg/sample). Endocannabinoid production in rat microglial cells, unlike that of other arachidonic acid metabolites, is not affected by lipoxygenase or cyclooxygenase inhibitors but production of 2-AG is greatly reduced by the diacylglycerol lipase inhibitor RHC80267. Conversely, addition of the ionophore A23187 enhances 2-AG production as a result of increased intracellular calcium (Fig. 1). We have also demonstrated, through PCR and immunoblotting, that these cultured microglial cells, which exist in a primed state, express the CB_2 cannabinoid receptor. This receptor is possibly expressed in a truncated form, judging by its reduced molecular weight. Localization by immunohistochemistry demonstrates that the CB_2 receptor is primarily found on intracellular membranes; we are conducting further studies to investigate whether this CB_2 intracellular localization changes with the activation state of microglia. These findings point toward endocannabinoid synthesis and release as autocoids in stable microglial cell cultures.

Fig. 1: Effects of a diacylglycerol lipase inhibitor and a calcium ionophore on 2-AG production



Acknowledgment: Supported by NIH grants DA08098 and DA09155.

RELEASE OF 2-ARACHIDONYLGLYCEROL (2-AG) FROM RBL-2H3 CELLS, A MODEL OF HUMAN MAST CELLS

Jay Chen, Bertold Vilner, Cheng Liu, and J. Michael Walker,
Brown UniversityDepartments of Psychology and
Neuroscience Providence, Rhode Island, 02912 USA

In spite of knowledge of the existence of endocannabinoids for many years, little work has directly shown their release from cells in the brain or in the periphery. In a previous study, we found that analgesia-inducing electrical stimulation of the periaqueductal gray produced increased levels of anandamide in microdialysates. Other work has shown that various treatments lead to increased membrane levels of endocannabinoids, but these studies did not directly demonstrate that the compounds are releasable.

We sought to determine whether various stimulatory agents would lead to release of 2-AG evidenced by increased levels of 2-AG in the medium of cultured RBL-2H3 cells. Cells were stimulated in three ways: 1) via the IgE receptor, 2) via direct activation of G proteins using compound 48/80 in quercetin-sensitized cells, or 3) via application of the calcium ionophore A23187. Following purification by reverse phase solid phase extraction columns, tandem mass spectrometry was used to quantify the levels of 2-AG. For purposes of comparison, leukotriene B4 (LTB4), leukotriene C4 (LTC4), leukotriene D4 (LTD4) and prostaglandin D2 (PGD2) were also measured.

All of the stimuli produced the release of 2-AG from RBL-2H3 cells. By contrast, PGD2 and the leukotrienes were elevated more strongly by the calcium ionophore than by stimulation with either IgE or by direct stimulation of the G-protein with compound 48/80 in quercetin sensitized cells. These findings indicate that upon stimulation, 2-AG is released from RBL-2H3 cells in amounts sufficient for further studies of the biochemical mechanisms that control its secretion.

MACROPHAGES LACKING THE CB_2 RECEPTOR DISPLAY ALTERED LEVELS OF IL1β, TNFα AND IL6 SECRETION

Tanya M. Ferguson, and Nancy E. Buckley
Department of Biological Sciences, California State Polytechnic University-Pomona; Pomona, CA 91768, USA

Macrophages participate in many events involving innate and acquired immunity to infection through the production and release of cytokines. Cannabinoids have been suggested to act as immunomodulators by altering such macrophage activity. Of particular interest is the modulation in production of acute phase cytokines interleukin-1β (IL1β), tumor necrosis factor alpha (TNFα), and interleukin-6 (IL6). Overall, the immunomodulatory mechanism by which cannabinoids alter macrophage production of cytokines is not clearly understood. However, the high levels of CB_2 receptor, and lack of CB_1 receptor, in macrophages suggests a CB_2 receptor involvement in immunoregulatory occurrences. In order to investigate the role of this receptor in cannabinoid-induced alterations in macrophage cytokine production, a unique mouse model is being used that is CB_2 receptor deficient. Thioglycollate elicited peritoneal macrophages in wild type and CB_2 knockout mice were stimulated with lipopolysaccharide to investigate the direct effect of cannabinoids (WIN55212-2, CP55940, HU210, and 2AG) at physiological concentrations on macrophage cytokine production. Cytokine secretion was measured using enzyme linked immunosorbent assay. One very striking result indicates that overall secretions of IL1β, TNFα, and IL6 are significantly decreased in macrophages obtained from the CB_2 knockout mice versus those obtained from wild type mice. Additionally, when comparing cytokine secretion levels in macrophages obtained from wild type and CB_2 knockout mice, IL1β secretion levels show an increase in wild type macrophages in the presence of cannabinoids and a decrease in CB_2 knockout macrophages. Secretions of TNFα by macrophages also reveal opposing results among wild type and CB_2 knockouts. In wild type macrophages, a decrease in secretion levels in the presence of cannabinoids is evident, with a possible biphasic effect taking place. In CB_2 knockout macrophages, TNFα secretions increase in the presence of cannabinoids and a biphasic effect is also seen here. The effect of cannabinoids on IL1β and TNFα secretions in CB_2 knockout macrophages suggest that a yet unknown third cannabinoid receptor may be mediating these effects. On the other hand, IL6 secreted levels measured from wild type and CB_2 knockout macrophages remain relatively unchanged in the presence of cannabinoids.

Our findings suggest that CB_2 receptor mediates the cannabinoid-induced effect on IL1β and TNFα secretion in macrophages of wild type mice. In addition, the reduced levels of IL1β, TNFα, and IL6 secretion in knockout macrophages suggest an important role of the cannabinoid system in cytokine production. Interestingly, we have found lower levels of anandamide in macrophages from CB_2 knockout as compared to wild type macrophages.

COMMERCIAL ANANDAMIDE (AEA) PREPARATIONS FROM DIFFERENT SUPPLIERS DIFFER IN THEIR EFFECTS ON THE RESPIRATORY BURST REACTION OF HUMAN NEUTROPHIL LEUKOCYTES (PMN)

Birgit Frommer, Hans G. Kress
Department of Anaesthesiology and Intensive Care Medicine (B),
University Hospital, Vienna, Austria

Abstract not published on the web at the request of the authors.

MODULATION OF INTERLEUKIN 2 (IL-2) RELEASE FROM PERIPHERAL BLOOD MONONUCLEAR CELLS BY CANNABINOIDS

Kenneth Ihenetu, Areles Molleman, Mike Parsons, and Cliff Whelan
University of Hertfordshire, College Lane, Hatfield, Herts. AL10 9AB, UK

Cannabinoids suppress the immune response by modulating cytokine release in a variety of immune cells (for review see Berdyshev, Chemistry and Physics of Lipids, **2000** 108, 169). The receptors mediating these events are poorly characterised. In this report, we have studied the effects of cannabinoid receptor agonists and antagonists on the release of interleukin 2 (IL-2) from peripheral blood mononuclear cells (PBMC).

PBMC were isolated from buffy coat blood using a modified technique originally developed by Boyum (Scand. J. Clin. Lab Invest, **1968**, 21, suppl. 97:77). PBMC were treated with CP55,940, Δ^9-THC or WIN55212-2 for 2h followed by stimulation with phytohaemagglutinin (PHA) 10μg ml^{-1} for another 18h. Cell culture supernatants were harvested and IL-2 measured by ELISA. In experiments where the effects of cannabinoid antagonists were studied, the cells were first incubated with SR141716A 10^{-6}M or SR144528 10^{-6}M for 30 minutes before the addition of the agonists. In experiments where the effects of CP55,940 on WIN55212-2 concentration effect curve were studied, CP55,940 (10^{-6}M) was added 30 minutes before the addition of WIN55212-2 (10^{-10}M – 10^{-5}M). Alcohol and dimethylsulphoxide were included at 0.1% concentration as vehicle controls. Results were analysed using Prism (Graph Pad Inc., San Diego California U.S.A). Values are presented as means ± S.E.M. or 95% confidence limits (CL) as appropriate. Students unpaired 't' test or one-way ANOVA was used to assess significant differences. Statistical significance was assumed if $P \leq 0.05$.

The purity and viability of PBMC were >97% and >95% respectively on all occasions. CP55,940 and Δ^9-THC (10^{-10}M-10^{-5}M) had no significant ($P > 0.05$) inhibitory effect on IL-2 release from PBMC whereas WIN55212-2 (10^{-10}M - 10^{-5}M) significantly ($P < 0.05$), inhibited IL-2 release from PBMC (IC_{50} = 9.3x10^{-7}M, 95% C.L = 8.1x10^{-7}M-1.1x10^{-6}M, n = 5). SR141716A (10^{-6}M) had no significant (P<0.05, n = 5) inhibitory effect on WIN55212-2 concentration effect curve (pA_2 >5, n=5) whereas SR144528 (10^{-6}M) significantly ($P < 0.05$) antagonised the inhibitory effect of WIN55212-2 (pA_2.value = 6.3 ± 0.1, n = 5). CP55,940 (10^{-6}M) antagonised the inhibitory effect of WIN55212-2 (pA_2 value = 6.1± 0.1, n=5).

We have shown that WIN55212-2 inhibits PHA-induced IL-2 release from PBMC. This effect was antagonised by SR144528 and CP55,940 but not SR141716A. Taken together, we suggest that WIN55212-2 suppresses immune response in PBMC by inhibiting IL-2 secretion via the CB_2 receptor. Furthermore, the antagonist effect of the CP55,940 in the present study indicates an affinity for, but low efficacy at CB_2 receptors in PBMC.

CB_1 AND CB_2 RECEPTOR PROTEIN EXPRESSION IN MOUSE IMMUNE CELL SUBPOPULATIONS

Thomas W. Klein, Kellie Larsen, Brian Lane, Liang Nong,
Ray Widen, Cathy Newton, and Herman Friedman
Department of Medical Microbiology amd Immunology,
University of South Florida, Tampa, Florida 33612, USA

Several groups have analyzed by Western blotting the expression of CBR protein in brain and immune cells. The molecular weight of CB_1 is reported to range from ~40 to 200 kDa in brain and immune cells while CB_2 ranges from ~27 to 40 kDa in immune cells. Similar studies on samples from mouse splenocyte subpopulations have not been reported and so in the current study we examined CB_1 and CB_2 receptor protein expression in these cell types. Fourteen amino acid peptides from the amino end of mouse CB_1 and the carboxy end of mouse CB_2 were conjugated to KLH and used for immunizing and boosting rabbits. High titer sera were identified based on ELISA reactivity to the peptide antigens and affinity purified by CBR peptide-sepharose chromatography. Preimmune sera, negative for ELISA reactivity, were also mock affinity purified. Purified antisera showed no reactivity to the heterologous CBR antigen. BALB/c splenocytes were isolated and fractionated into plastic adherent cells, non-adherent cells, as well as B cells and T cells enriched by anti-Ig panning. Mouse cell lines, N18TG2 neuroblastoma and JAWSII dendritic cell, and the human T cell line Jurkat were also used. Cellular protein was isolated and analyzed by Western blotting. Results with CB_1 antibody showed the major band occurred at 76-79 kDa in all immune cell subpopulations with the exception of JAWSII dendritic cells. The major band for N18 was at 67 kDa with several minor smaller bands. Probing with the corresponding preimmune serum showed only one band at ~60 kDa in Jurkat preparations. Results with CB_2 antibody showed two major bands, one ~ 50 kDa and the other ~45 kDa in all immune cell subpopulations. N18 cells showed several major bands ranging in size from ~55 to ~30 kDa. Probing with the corresponding preimmune serum showed one band at ~70 kDa in Jurkat and N18. Regarding the relative quantities of receptor protein among the various cell subpopulations, the trend was for non-adherent splenocytes to display more than adherent cells, and for B cells to display more than T cells. JAWSII were negative for CB_1 expression and N18 cells expressed either multiple isoforms of CB_2 or of a cross-reacting protein. These results suggest that mouse splenocyte subpopulations express both CB_1 and CB_2 proteins with the former showing one isoform at 76 to 79 kDa while the latter showing two isoforms at 45 and 50 kDa. Interestingly, N18 cells appear to express CB_2 in addition to CB_1 and dendritic cells appear to express only CB_2. The relative quantities of CB_1 and CB_2 among the various immune subpopulations observed suggests that non-adherent cells express more protein than adherent cells and B cells express more than T cells. Further studies are needed to determine if CBR proteins expressed by immune cells differ from those expressed by other tissues.

Acknowledgements: Supported by NIDA grants DA10683, DA03646, and DA07245

Δ^9-TETRAHYDROCANNABINOL (Δ^9-THC) AND CP55940 MEDIATED INHIBITION OF INTERLEUKIN-2: DIFFERING MECHANISMS OF ACTION IN HUMAN T CELLS

Gautham K. Rao, and Norbert E. Kaminski
Department of Pharmacology and Toxicology,
Michigan State University, East Lansing, MI 48824 USA

Cannabinoid compounds are well known modulators of leukocyte function. Previous studies from this laboratory have shown T cells to be a sensitive target to cannabinoid treatment. In particular, cannabinoids inhibit the production of interleukin (IL)-2 by T cells. In the present studies, HPB-ALL and Jurkat E6-1 human T cell lines were used to elucidate the mechanism of IL-2 inhibition by Δ^9-THC, a plant-derived cannabinoid, and CP55940, a synthetic cannabinoid. While both cells lack CB_1 receptor transcripts, previous investigation has revealed through Northern blot analysis that the CB_2 receptor transcript in HPB-ALL is 2.4kb (as previously reported), but aberrant in Jurkat E6-1 cells as evidenced by the identification of a 2.3, 4.5, and 5.7kb transcripts. The objective of the present studies was to examine the effect of Δ^9-THC and CP55940 on intracellular calcium ($[Ca^{2+}]_i$) and inhibition of phorbol ester (PMA) and ionomycin (Io) induced IL-2 secretion in HPB-ALL and Jurkat E6-1 cells. Δ^9-THC (10μM) induced a sharp rise (500-700nM) in $[Ca^{2+}]_i$ in HPB-ALL cells as compared to a modest rise (80-100nM) in Jurkat E6-1 cells. This rise in $[Ca^{2+}]_i$ was antagonized in the presence of the CB_2 receptor antagonist, SR144528 (1μM), in HPB-ALL cells, but not in Jurkat E6-1 cells. Accordingly, Δ^9-THC inhibited IL-2 secretion in HPB-ALL cells in a concentration dependent manner, but not in Jurkat E6-1. CP55940 (10μM), on the other hand, failed to induce a rise in $[Ca^{2+}]_i$ in either cell type, but exhibited concentration dependent inhibition of IL-2 secretion in both cell types. These data suggest that Δ^9-THC and CP55940 may act to modulate IL-2 secretion through different mechanisms of action in human T cells.

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THE PUTATIVE ENDOGENOUS CANNABINOID, 2-ARACHIDONYL GLYCEROL, CAUSES AN INHIBITION OF THE T CELL GROWTH FACTOR, INTERLEUKIN-2, AND A PARADOXICAL AND CONCURRENT ENHANCEMENT OF T CELL PROLIFERATION

Cheryl Rockwell, and Norbert Kaminski
Department of Pharmacology and Toxicology,
Michigan State University, East Lansing, MI, 48824 USA

2-arachidonyl glycerol (2-AG), is an arachidonic acid derivative that has been shown through radioligand binding studies to bind to both cannabinoid receptors, CB_1 and CB_2. 2-AG mimics some of the immunomodulatory effects of the plant-derived cannabinoids, delta^9-THC and cannabinol, including inhibition of secretion of several different cytokines, such as IL-2, IL-4, and IFN-gamma. The first objective of the present studies was to determine the role of the CB_1 and CB_2 receptors in 2-AG-mediated inhibition of IL-2. IL-2 was measured from cell supernatants by ELISA. In activated murine splenic and thymic T cells, 2-AG causes a marked and concentration-responsive inhibition of IL-2 that is more potent than anandamide, another putative endogenous cannabinoid. The CB_1 and CB_2 antagonists, SR141716A and SR144528 respectively, did not antagonize the inhibition of IL-2 mediated by 2-AG or anandamide. Because arachidonic acid also inhibits IL-2 in a concentration-dependent manner, a second objective was to ascertain the roles of fatty acid amidohydrolase (FAAH), the anandamide transporter (AMT), and cyclooxgenase (COX) in the inhibition of IL-2 by 2-AG. Neither methyl arachidonyl fluorophosphonate (MAFP), an inhibitor of FAAH, nor AM404, an inhibitor of AMT, reversed the inhibitory effect of 2-AG on IL-2 secretion. Pretreatment with nonselective COX inhibitors, such as acetyl salicylic acid, did not alter the activity of 2-AG on IL-2 secretion. The third objective of the present studies was to determine the effect of 2-AG upon T cell proliferation as measured by ^{3}H-thymidine incorporation. Interestingly, 2-AG causes a concurrent enhancement of T cell proliferation at the same concentrations at which it inhibits the production of IL-2. Neither anandamide nor arachidonic acid caused an enhancement of T cell proliferation. These data suggest that 2-AG enhances T cell proliferation while paradoxically and concurrently inhibiting the production of the T cell growth factor, IL-2, in activated murine splenic and thymic T cells. These data also suggest that the 2-AG-mediated enhancement of T cell proliferation is unique to 2-AG and not common to arachidonic acid and its derivatives.

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ATP SELECTIVELY INCREASES 2-ARACHIDONYLGLYCEROL PRODUCTION FROM CULTURED MICROGLIA

Anke Witting[1], Allyn Franklin[1], and Nephi Stella[1,2].
[1]Department of Pharmacology, and [2]Department of Psychiatry and Behavioral Sciences,
University of Washington, Seattle, WA 98195-7280, USA

Virtually all types of brain insults rapidly activate microglia, the tissue macrophages of the brain. Substances released by damaged cells, such as large amounts of the nucleotide ATP, are believed to participate in the activation of microglia; yet the molecular mechanisms that underlie this activation process are poorly understood.

Because exogenous and endogenous cannabinoids have been shown to have immunomodulatory effects on microglia, we hypothesized that these cells produce endocannabinoids and that this production is affected by ATP-induced activation.

We used primary mouse microglia and measured the production of endocannabinoids by gas chromatography/mass spectrometry; namely the two well-known endocannabinoids anandamide and 2-arachidonylglycerol (2-AG), as well as the two endocannabinoid candidates homo-γ-linolenylethanolamide (HEA) and docosatetraenylethanolamide (DEA).

Unstimulated microglia produced detectable amounts of all four endocannabinoids. Stimulation of microglia with ATP selectively increased the production of 2-AG in a dose- and time-dependent manner. The production was due to P2X7 receptors since the agonist 3'-O-(4-benzoyl)benzoyl ATP (Bz-ATP) increased the production of 2-AG, while the antagonist oxidized ATP abolished the ATP induced production of 2-AG.

Our results demonstrate that primary mouse microglia synthesize at least four endocannabinoids and that ATP selectively increases the production of 2-AG by activating ionotropic purinergic P2X7 receptors. They also suggest that the biochemical pathway leading to the production of 2-AG is separate from the ones leading to anandamide, HEA and DEA. Endocannabinoids may participate in the mechanisms controlling microglia activation.

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(S)-AMPA INHIBITION OF ELECTRICALLY EVOKED CALCITONIN GENE-RELATED PEPTIDE (CGRP) RELEASE FROM THE RAT DORSAL HORN: REVERSAL BY CANNABINOID ANTAGONIST SR141716A

Jason W. Brooks[1,2], Stephen W.N. Thompson[2], Andrew S. Rice[1], and Marzia Malcangio[2]
[1]Pain Research, Imperial College, London, United Kingdom. [2]Centre for Neuroscience Research, Kings College, London, United Kingdom

Previous studies have identified both cannabinoid (CB_1) receptors and endogenous agonists in the spinal cord. Additional *in vitro* studies suggest that endocannabinoids are synthesized on 'demand' following cell depolarisation (Di Marzo et al, Nature, **1994**, 372, 686-691). In the present study, we have investigated the effect of both a CB_1 agonist (WIN55,212-2) and and an AMPA receptor agonist on electrically evoked CGRP release in the rat dorsal horn.

Horizontal lumbar dorsal horn slices (Wistar rats, 200-270g) with dorsal roots (L4-L5), were mounted in a 3-compartment bath and superfused with modified oxygenated Krebs' solution at room temperature (Malcangio M & Bowery N.G, Pain, **1996**, 66, 351-358). Nine 8-min fractions were collected in the following order: (1-3) basal outflow,(4) +/- drug superfusion, (5) electrical stimulation (20V, 0.5msec, 10 Hz) +/- drug superfusion and (6-9) recovery fractions. Compounds were dissolved in either 100% DMSO or distilled water and diluted to the required concentration with modified Krebs' solution. CGRP-like immunoreactivity (CGRP-LI) content in the concentrated perfusates was determined by a specific radioimmunoassay using the scintillation proximity bead assay technique (sensitivity 1fmol/tube).

Basal outflow CGRP-LI from control slices was 25.4 ± 2.5 fmol/8ml fraction (mean ± s.e.m. n=8) and increased to 58.7 ± 8.8 fmol/fraction during electrical stimulation ($p<0.05$). WIN55,212-2 (100nM & 1μM) significantly inhibited electrically evoked CGRP-LI release, which was antagonised by co-superfusion of SR141716A (CB_1 receptor antagonist-5μM). (S)-AMPA (5μM) superfusion inhibited electrically evoked-CGRP-LI release compared to control, which was antagonised by co-superfusion with SR141716A (5μM) but not the $GABA_B$ receptor antagonist (CGP55485-1μM).

Our data shows that activation of AMPA receptors in the dorsal horn inhibited CGRP release from activated primary afferent fibres. Since the effect of AMPA was antagonized by SR141716A, we suggest that AMPA induced production of endogenous cannabinoids in second order neurons retrogradely inhibits activity-induced release of sensory neuron transmitters.

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CHARACTERIZATION OF THE THERAPEUTIC ANTI-INFLAMMATORY EFFECT OF PALMITOYLETHANOLAMIDE IN A RAT MODEL OF ACUTE INFLAMMATION

Silvia Conti[1], Barbara Costa[1], Mariapia Colleoni[2], and Gabriella Giagnoni[1]
[1]Dipartimento di Biotecnologie e Bioscienze, Università di Milano-Bicocca, 20126 Milano, Italy; [2]Dipartimento di Farmacologia Chemioterapia e Tossicologia Medica, Università di Milano, 20129 Milano, Italy

Our previous results showed that the endocannabinoid palmitoylethanolamide (PEA) had anti-inflammatory properties in the rat carrageenan-induced model of acute inflammation, when orally administered, at the dose of 10 mg kg^{-1}, before the induction of inflammation. The specific CB_2 receptor antagonist SR144528 (3 mg kg^{-1}, p.o., 1h before PEA) reversed this anti-inflammatory effect of PEA, suggesting a possible involvement of this receptor (Conti S et al., Br. J. Pharmacol. **2002**, 135, 181-187). More recently, we also demonstrated that the same dose of PEA was able to reduce the paw edema when daily administered after the onset of inflammation, for 4 days. This PEA effect was associated with its ability to bring free radicals and nitrite/nitrate tissue levels down to those found in non inflamed paw tissue; on the contrary cyclooxygenase (COX) system seemed not to be involved, since both COX activity and serum prostaglandins (PG) were still increased after PEA treatment. Since our previous biochemical results could be affected by the presence of PEA administered the last day of treatment (1 h just before sacrifice), in the present work, rats did not receive the last administration of the drug and all the behavioural and biochemical parameters have been re-evaluated. After the daily administration of increasing doses of PEA (1,3,5 and 10 mg kg^{-1}), paw edema decreased in a time- and dose- dependent manner, so suggesting a possible receptor-mediated mechanism. Repeated treatment with SR144528 (1 and 3 mg kg^{-1}, p.o., for 3 days, 1 h before PEA administration) did not reverse the anti-inflammatory effect of 10 mg kg^{-1} PEA, so showing that CB_2 receptor was not involved in the therapeutic effect of PEA. The two antagonist doses were *per se* ineffective. Future aim will be to study the mediation by other receptors, such as vanilloid, CB_1 receptors, etc...

The inhibition of nitric oxide and radical oxygen production induced by PEA was still evident also without the last admnistration, and it was also dose-related. On the contrary the presence of PEA due to its administration also the fourth day strongly affected the COX system. In fact, following only three administrations of PEA, COX activity and serum PG levels were decreased in comparison with inflamed control animals. Furthermore these PEA effects showed a dose-relation. To date the specific target of this endocannabinoid remains unknown, even if our future studies will be addressed to better define whether PEA, as classical non steroidal anti-inflammatory drugs, acts specifically on COX system and whether is selective for a COX specific isoform: COX-1 or COX-2.

THE ANTINOCICEPTIVE EFFECT OF THC INVOLVES MODULATION OF ENDOGENOUS OPIOIDS

Melinda Cox, and Sandra Welch
Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, Virginia 23298, USA

Δ^9-THC (THC) is the major psychoactive constituent of *Cannabis sativa*, and produces hypothermia, analgesia, catalepsy, and hypoactivity. THC acts via CB_1 and CB_2 receptors, which are blocked by SR141716A and SR144528, respectively. There are three types of opioid receptors - mu, kappa, and delta. Antagonists to these include naloxone (non-selective), nor-BNI (kappa), and naltrindole (delta). Several lines of evidence have demonstrated interactions between cannabinoids and kappa opioids. We addressed the hypothesis that THC-induced release of endogenous opioids results in antinociception in arthritic and normal rats. Morphine is an opioid that is used to treat chronic pain. It produces antinociception through its actions at the mu opioid receptor. We further hypothesize that synergy between THC and morphine may be useful for combination therapy in the treatment of chronic pain. Opioids are the current treatment of choice for chronic pain, yet tolerance and side effects develop. Thus, the mechanisms underlying such combination-induced synergy need to be evaluated. Using the Freund's Adjuvant-Induced Arthritis (FAA) test via the induction of arthritis by mycobacterial adjuvant, male Sprague-Dawley rats were rendered arthritic in 19 days. The rats were tested for antinociceptive effects of THC (nonarthritic ED_{50} = 2.1mg/kg; arthritic ED_{50} = 2.5mg/kg) or morphine (nonarthritic ED_{50} = 2.4mg/kg; arthritic ED_{50} = 2.2mg/kg) using the paw pressure test. Antagonists, naloxone (1, 5 mg/kg, s.c.), naltrindole (2mg/kg, s.c.), nor-BNI (2, 10 mg/kg, s.c.), and SR141716A (10mg/kg, i.p.) were evaluated for their abilities to block antinociception produced by morphine or THC. Spinal perfusion 10 and 30 min following injection with vehicle or THC (4, 5mg/kg, i.p.) was performed to collect CSF for measurement of endogenous opioid peptides using radioimmunoassays. Our results indicate that morphine and THC are equally potent and efficacious in nonarthritic and FAA rats in the paw pressure test. Morphine-induced antinociception is attenuated by naloxone in nonarthritic and FAA rats. THC-induced antinociception is attenuated by SR141716A and naloxone in both treatment groups. Naltrindole fails to attenuate THC-induced antinociception in either non-arthritic or FAA rats. THC-induced antinociception is attenuated by nor-BNI in FAA rats only. THC (i.p.) does not release dynorphin A (dyn A), leu-enkephalin (leu-enk), dynorphin B, or met-enkephalin 30 min post-administration in nonarthritic or FAA rats. At 10 min following THC injection, dyn A levels are increased in nonarthritic rats. At 10 min post-vehicle administration, dyn A levels are increased in FAA rats. Leu-enkephalin levels are significantly elevated in nonarthritic rats 10 min after THC administration, while levels are slightly decreased in FAA rats. It is hypothesized that the elevated dyn A level in FAA rats contributes to hyperalgesia by dynorphin interaction with NMDA receptors and that THC induces antinociception by decreasing dyn A release. Thus, the interaction of dyn A with the kappa opioid receptor predominates, resulting in antinociception.

INHIBITION OF TNFα PRODUCTION BY THE CANNABINOID CB_1 RECEPTOR ANTAGONIST SR141716 MAY ACCOUNT FOR ITS PREVENTION OF INDOMETHACIN INTESTINAL INJURY IN RATS

Tiziano Croci[1], Marco Landi[1], Pietro Marini[1],
Murielle Rinaldi-Carmona[2], and Luciano Manara[1]
[1]Research Center Sanofi Midy – Sanofi Synthelabo S.p.A., Via G.B. Piranesi 38, 20137 Milan, Italy, [2]Sanofi Synthelabo, 371 rue du Pr. J. Blayac, 34184 Montpellier, France

TNFα has been suggested to play a major role in the early stage of nonsteroidal anti-inflammatory agent's toxicity for the small intestine (Bertrand *et al., Br. J. Pharmacol* **1998**, *124*, 1385).

We investigated the involvement of TNFα in intestinal lesions by indomethacin in rats and in their prevention by the cannabinoid CB_1 receptor antagonist SR141716 (Rinaldi-Carmona *et al.*, FEBS Lett. **1994**, *350*, 240). Overnight fasted male CD rats, 220-260 g., received E. coli lipopolysaccharide (LPS) 1 mg/kg, i.v., 90 min, or indomethacin, 20 mg/kg, s.c., 24 h before the sacrifice; SR141716, 1-10 mg/kg p.o. was given 2h before LPS or 6h after indomethacin. TNFα in plasma of vehicle or LPS treated rats was, pg/ml means ± se, 30 ± 20 and 3,190 ± 200 respectively. Number of ulcers in the jeunum-ileum, myeloperoxidase activity (MPO) and TNFα tissue levels of vehicle or indomethacin treated rats, were as listed: 0 and 110 ± 11; 18 ± 11 and 221 ± 20, pg/mg protein; 170 ± 20 and 690 ± 77 μU/mg protein. SR141716, that had no influence on the aforementioned endpoints, by itself, dose-dependently inhibited LPS (ID_{50}, 1 mg/kg) and indomethacin (ID_{50}, 2.4, 2.0 and 1.4 mg/kg) effects. Our findings provide unequivocal evidence of potent inhibition of TNFα production by SR141716 that may account for its ability to protect rats from indomethacin intestinal injury. Whether cannabinoid CB_1 receptors are involved in this action of SR141716 remains to be established.

ACTIONS OF ANANDAMIDE ON CULTURED SENSORY NEURONES

Rhian M. Evans, Roderick H. Scott, and Ruth Ross
Department of Biomedical Science, IMS, University of Aberdeen,
Foresterhill, Aberdeen AB25 2ZD, UK

We have previously identified cannabinoid receptor-mediated responses in cultured dorsal root ganglion (DRG) neurones from neonatal rats (Ross et al. Neuropharmacology **2001**, 40: 221-232). The aims of the present study were to characterise the effects of anandamide (arachidonylethanolamide: AEA) on high voltage-activated Ca^{2+} currents (I_{Ca}), K^+ -evoked and vanilloid receptor-mediated Ca^{2+} flux. DRG neurones were maintained in culture in the presence of 20ng/ml NGF. The whole cell recording technique was used to investigate I_{Ca} activated by 100ms depolarisations to 0 mV from a holding potential of –90 mV. The neurones were bathed in choline chloride-based extracellular solution (in mM: 130 choline chloride, 2 $CaCl_2$, 3 KCl, 0.6 $MgCl_2$, 1 $NaHCO_3$, 10 HEPES, 5 glucose, 25 tetrethylammonium chloride, 0.0025 tetrodotoxin). Patch pipettes were filled with CsCl-based solution (in mM: 140 CsCl, 0.1 $CaCl_2$, 5 EGTA, 2 $MgCl_2$, 2 ATP, 10 HEPES). Ca^{2+}-imaging experiments were performed on neurones loaded with fura-2 and stimulated by sequentially applying, capsaicin (10-100nM), AEA (0.1-10 μM) and NaCl-based solution containing 30mM KCl. AEA and capsaicin were made up in DMSO to give a final concentration of 0.01% and in all experiments the neurones were continually bathed in extracellular solutions containing 0.01% DMSO. 0.01% DMSO was not found to significantly modulate I_{Ca}. Three to five minutes extracellular application of 100nM AEA reduced the peak I_{Ca} from –1.01 ± 0.12 nA to –0.77 ± 0.11 nA (mean ± sem n=8; $P<0.02$ student's t-test). Partial recovery of the I_{Ca} was observed in only 2 out of the 8 neurones. Consistent with these results we found that Ca^{2+} transients evoked by KCl were also attenuated by AEA (1 μM). The total Ca^{2+} flux was reduced in the presence of AEA by 25 ± 6 % (n=29; $P<0.0003$) and partial but significant ($P<0.05$) recovery to 87 % of the control value was observed. Capsaicin (1 μM) evoked transient inward currents that desensitized. In Ca^{2+} imaging experiments 10 and 100 nM capsaicin induced transient Ca^{2+} influx in 10 (n=24) and 76 % (n=62) of neurones studied respectively. These Ca^{2+} transients were not attenuated by thapsigargin (500 nM) but were not activated when capsaicin was applied in a Ca^{2+}-free extracellular solution. In these same experiments AEA (1 μM) was applied before capsaicin and Ca^{2+} transients were evoked in 6 out of 62 neurones. Only 1 neurone responded to AEA but not subsequently to capsaicin. The responses to 100 nM capsaicin were significantly larger than those produced by 1 μM AEA with fluorescence ratio values 2.27 ± 0.28 (n=47) compared to 0.54 ± 0.25 (n=6). Changing the order of drug application so that capsaicin was applied before AEA resulted in 69 % (n= 29 of 42 neurones) responding to capsaicin and 31 % of neurones responding to AEA (n=13 of 42 neurones). AEA was found to evoke responses in four neurones, which previously did not respond to capsaicin. Analysis of the size of neurones that responded to capsaicin but did not respond to AEA, showed that this population of capsaicin-sensitive neurones were significantly larger than neurones which were sensitive to both drugs. In conclusion AEA inhibited Ca^{2+} influx through voltage-activated channels in DRG neurones but activated a cation channel that was permeable to Ca^{2+} in a subpopulation of neurones. Our data suggests that AEA may act on several distinct receptors to modulate conductances in cultured DRG neurones.

pH DEPENDENT INHIBITION OF FATTY ACID AMIDE HYDROLASE BY NON-STEROIDAL ANTI-INFLAMMATORY DRUGS: COMPARISON OF NON-SELECTIVE AND CYCLOOXYGENASE 2-SELECTIVE COMPOUNDS

Sandra Holt, Gunnar Tiger, and Christopher J. Fowler
Department of Pharmacology and Clinical Neuroscience,
Umeå University, SE-901 87 Umeå, Sweden

It is now well established that non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, indomethacin and flurbiprofen inhibit the metabolism of anandamide and palmitoylethanolamide by fatty acid amide hydrolase (FAAH) at therapeutically relevant concentrations (Paria *et al., Mol. Reprod. Dev.* 45 [**1996**] 183-92; Fowler *et al., J. Pharmacol. Exp. Ther.* 283 [**1997**] 729-34; Fowler *et al., Arch. Biochem. Biophys.* 362 [**1999**] 191-6). Recently, we found that a reduction in the assay pH increased the sensitivity of FAAH to inhibition by ibuprofen (Holt *et al., Br. J. Pharmacol.* 133 [**2001**] 513-20). In the present study, we have investigated a) whether the increased sensitivity to ibuprofen is seen with other NSAIDs; and b) whether reductions in extra- or intra-cellular pH affect the sensitivity to ibuprofen in intact cells.

Using homogenates from rat brain (minus cerebellum) indomethacin and the (R)- and (S)-enantiomers of flurbiprofen nhibited FAAH with IC_{50} values of 54, 97 and 50 μM, respectively, at an assay pH of 8. When the assay pH was reduced to 6, the IC_{50} values were reduced to 17, 31 and 13 μM, respectively. This pH sensitivity was also seen with the COX-2 selective inhibitor niflumic acid, IC_{50} values of 370 and 67 μM being seen at pH 8 and 6, respectively. In contrast, celecoxib showed no pH dependence (IC_{50} values ~300 μM at both pH values), and nimesulide and SC-58125 (COX-2 selective inhibitors like celecoxib) did not inhibit FAAH.

In intact C6 glioma cells, a reduction in the extracellular pH increased the sensitivity of the FAAH to inhibition by ibuprofen. Thus, for example, the inhibition of metabolism of exogenously added anandamide in the intact cells produced by 100 μM (-)ibuprofen was 24, 52 and 72% at extracellular pH values of 7.4, 6.8 and 6.2, respectively. Indeed, at pH 6.2, a concentration of (-)ibuprofen as low as 10 μM reduced the rate of anandamide metabolism by 38%. A selective reduction in the intracellular pH was achieved by preincubation of the cells with ammonium chloride followed by medium change to sodium-free buffer at pH 7.4. Under these conditions, a reduction in the intracellular pH did not affect the sensitivity of anandamide hydrolysis to inhibition by (-)ibuprofen.

Taken together, these data suggest that the unionised forms of indomethacin, flurbiprofen and ibuprofen are responsible for the inhibition of FAAH. In intact cells, the dependence of the extracellular, but not intracellular pH upon the sensitivity to inhibition by (-)ibu-profen would suggest that the degree of ionisation of the NSAID regulates its ability to enter the cell. However, given that a reduction in the extracellular pH is seen in inflammation, these data would suggest that FAAH is more likely to be inhibited by ibuprofen in inflamed than in normal tissue.

SUPPRESSION OF INFLAMMATORY AND NEUROPATHIC PAIN BY CT-3: SITE OF ACTION, MOLECULAR AND PHARMACOKINETIC MECHANISMS

Susan M. Huang, Susan H. Imamura, Timothy J. Petros, Karen W. Petek,
Heather B. Bradshaw, and J. Michael Walker
Departments of Psychology and Neuroscience, Brown University,
Providence, RI 02912, USA

1,1-Dimethylheptyl-Δ^8-THC-11-oic acid (CT-3 or ajulemic acid) is a potent analog of THC-11-oic acid (the metabolite of THC). CT-3 was shown to have anti-inflammatory actions, analgesic properties in acute pain, and have effects on tumor cell growth. Throughout these studies, it was notable that therapeutic effects could be achieved without invoking gross side effects such as catalepsy and hypomotility. This apparent lack of psychotropic effects of CT-3 has distinguished it from typical cannabinoids, making it an attractive candidate for therapeutic development. Here we extended the characterization of CT-3 to models of chronic inflammatory pain and neuropathic pain in rats, and investigated the mechanisms that contribute to the unique behavioral profile of CT-3.

(1) The systemic effect of CT-3 was tested on models of chronic inflammatory (induced by Complete Freund's Adjuvant) and neuropathic pain in rats. CT-3 was effective in suppressing thermal hyperalgesia in CFA-inflamed rats, and it reversed the mechanical allodynia characteristic of peripheral neuropathy.

(2) To investigate the sites of action of CT-3, we administered the drug locally in CFA-inflamed rats. Peripheral administration of CT-3 (i.pl.) and intracerebral (i.c.v.) administration were effective in suppressing CFA-induced hyperalgesia. However, administration via the intrathecal (i.t.) route was ineffective.

(3) In competition binding with [^{3}H]SR141716A, CT-3 exhibited an approximate 4 fold higher Ki in spinal cord compared to brain membrane preparations. Likewise, in [S^{35}]GTPγS binding assays, CT-3 behaved as a partial agonist in the brain in comparison to CP55,940, and it failed to produce G-protein stimulation in spinal cord membranes (up to 10μM).

(4) To test the possibility of variation in drug redistribution, brain and spinal cord levels of CT-3 and Δ^9-THC 30min following intraperitoneal (i.p.) injections of equimolar amounts were analyzed. The proportion of CT-3 present in the brain was considerably less compared to that seen with Δ^9-THC.

In summary: CT-3 was found to be effective in animal models of chronic inflammatory and neuropathic pain. The limited redistribution to brain centers and the partial agonist action could contribute to the lack of psychoactivity of CT-3. The lack of effect of CT-3 in the spinal cord may indicate variations in receptor reserve between the spinal cord and the brain. The above results also point to the periphery as an important site of action of CT-3.

INTRA-PERIAQUEDUCTAL GREY ADMINISTRATION OF THE CANNABINOID AGONIST HU210 REDUCES FORMALIN-INDUCED PAIN IN THE LATE PHASE

Maulik Jhaveri, David Finn, Simon Beckett, David Kendall,
Charles Marsden, and Victoria Chapman
School of Biomedical Sciences, University of Nottingham, Nottinghamshire, UK

The midbrain periaqueductal grey (PAG) is a part of the descending pain pathway. Cannabinoid CB_1 receptors are expressed in the PAG (Herkenham *et al., J. Neurosci.* **1991,** *11*, 563-583) and administration of the cannabinoid agonist WIN55212-2 into the dorsal PAG or CP55940 (Lichtman *et al., J. Pharmacol. Exp. Ther.* **1996***, 276*, 585-593) into the ventrolateral PAG induces analgesia in the rat tail-flick test. This study aimed to investigate the effects of HU210 in the dorsal PAG and its modulation of formalin-induced pain behaviour in rats.

A guide cannula was implanted (AP -6.7, ML 1.7, DV -3.6mm relative to bregma and skull surface, angle of 20^o) in male Sprague-Dawley rats (225-275g) under isoflurane/N_2O/O_2 anaesthesia. Formalin (50µl, 2.5% in 0.9%NaCl) was injected in the plantar surface of the rat right hind paw 10min after HU210 (0.1, 1 or 5µg in 250nl, n=4-5) or vehicle (250nl 60% DMSO in aCSF, n=4) microinjection in the dorsal PAG. Distance moved, rearing, grooming, and pain behaviours of the rat were scored in a Plexiglas box using Ethovision software. The pain behaviours were categorised as injected paw above the floor (C1), and licking, biting, holding or shaking/flinching of paw (C2). A composite pain score (CPS, Watson *et al., Pain* **1997,** *70*, 53-58) was calculated ([duration in C1 + (2*duration in C2)]/duration of trial). The data were analysed using ANOVA and Bonferroni/Dunn post-hoc test.

HU210 did not alter the distance moved or rearing behaviour of rats when compared to controls in the pre-formalin trial but significantly ($p<0.05$) reduced grooming at the 1µg dose. The first 5min (early phase) and 25-30min (late phase) marked two distinct phases of pain behaviour following formalin injection in control rats. HU210 (5µg) significantly ($P<0.001$) reduced pain behaviour in the late phase (Figure 1).

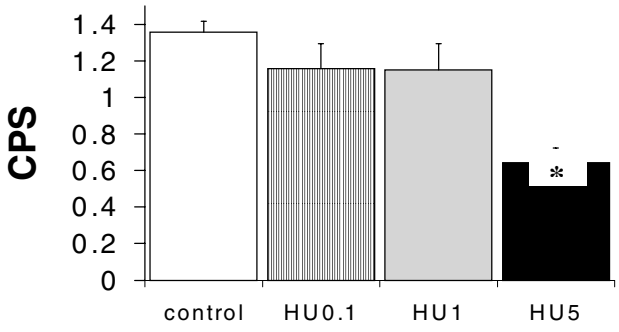

Figure 1 **:** *Effects of HU210 (HU, 0.1, 1 and 5µg in 250nl) on the composite pain score (CPS) in the late phase of formalin-induced pain behaviour. Each bar represents mean±SEM (n=4-5). * p<0.001 ANOVA followed by Bonferroni/Dunn post-hoc.*

These data indicate that cannabinoid receptor stimulation in the PAG reduces pain behaviour in the late phase but not the early phase of formalin response. This work supports the role of cannabinoid CB_1 receptors in analgesia mediated via the descending inhibitory pain control pathway. Further studies are required to confirm the CB_1 receptor involvement using selective CB_1 receptor antagonist, SR141716A.

COMPARISON OF PGF2α, AGN 192024 (PROSTAMIDE) and BUTAPROST (EP2 AGONIST) ON CTGF AND Cyr 61 mRNA EXPRESSION

Yanbin Liang, Chen Li, and David F. Woodward
Allergan, Inc., 2525 Dupont Dr. Irvine, CA 92612

Connective tissue growth factor (CTGF) and cysteine-rich angiogenic protein 61 (Cyr61) are the members of the CCN gene family that encodes multifunctional, extracellular matrix-associated signaling proteins that regulate cell adhesion, migration, proliferation, survival, and differentiation. Using Affymetrix gene chip technology, we first identified that prostaglandin F2a dramatically upregulated Cyr61 and CTGF mRNA expression in the HEK 293/EBNA cells (hFP-HEK 293/EBNA) stably expressing the human FP receptor. Using real time RT-PCR and Northern blot analysis, we further confirmed that PGF2a-induced upregulation of Cyr61 and CTGF mRNA expression is dose- and time- dependent. The PGF2a-induced upregulation of Cyr61 mRNA could be blocked by Rho inhibitor (Toxin B), whereas PGF2a-induced CTGF upregulation could be blocked by Rho inhibitor, PKC inhibitor (GF109203) and MAPK inhibitor(PD980159). This suggests that PGF2a-induced upregulation of Cyr61 is via Rho pathway, and the upregulation of CTGF is via multiple intracellular pathways. In an isolated cat iris tissue bath study, we found that both PGF2a and AGN 192024 triggered cat iris smooth muscle contraction at very similar EC50 (11 nM for PGF2a; 57 nM for AGN192024). Further gene expression studies revealed that both PGF2a and AGN 192024 upregulated Cyr61 mRNA expression in the cat iris. Only PGF2a, but not AGN 192024, upregulated CTGF mRNA expression in the cat iris. Therefore, PGF2a and AGN 192024 appear to interact with different receptors in cat iris. In a comparison with the prostaglandin FP receptor (Gq coupled), activation of prostaglandin EP2 receptor (Gs coupled) with butaprost also upregulated Cyr61 and CTGF mRNA expression in the isolated cat iris. Thus, despite quite different signal transduction pathways, both FP and EP2 receptor stimulation upregulates CTGF and Cyr 61. The prostamide analog AGN 192024 affected only Cyr61.

CANNABINOIDERGIC MODULATION OF SPINAL SUBSTANCE P RELEASE IN THE RAT

Jason B. Schechter, and Sandra P. Welch
Virginia Commonwealth University, Pharmacology and Toxicology Department, Richmond, VA 23298, USA

Carrageenan-induced inflammation is a commonly utilized animal model for the study of human edema development and pain (Yu *et al.*, **1998**). Carrageenan is a vegetable-derived gelatin isolated from Irish moss (*Gigartina aciculaire* and *G. pisillata)*. Type IV lambda (λ) carrageenan, in particular, is a non-gelling form – that is, it will not pass into a liquid-solid (gel) phase at room/physiological temperatures. Intraplantar administration of 50μL 1% λ-carrageenan (w/v; in 0.9% sterile saline) produces a localized inflammatory response evidenced by significant volumetric increase of the ipsilateral paw. The resulting vasodilation and microvascular leakage results in subcutaneous edema and is behaviorally manifested as a hyperalgesic guarding of the paw. Following unilateral and/or bilateral subcutaneous intraplantar bolus carrageenan injection, the resulting inflammation has proven to be an acute spatially restricted noxious edematous effect that engenders the release of eicosanoid inflammatory mediators. However, the resulting ascending pain messages, as relayed by the tachykinin messenger Substance P, have not yet been quantified.

Substance P represents the best-delineated member of a novel neurotransmitter system - a family of 3 related peptides known as neurokinins. The others are known simply as neurokinin A and neurokinin B. The specific receptor subtypes that correspond to these neurokinins are neurokinin 1 receptors for Substance P, neurokinin 2 receptors for neurokinin A, and neurokinin 3 receptors for neurokinin B. These neurotransmitters appear to play a key behavioral role in the regulation of emotions, and antagonists of their receptors may represent novel psychotropic drugs of the future (Stahl, **1999**).

The present research hopes to further delineate and quantify the release and behavioral effect characteristics of tachykinin messengers of pain by quantification of spinal release following peripheral subcutaneous insult. In studies of rheumatoid arthritis, the human synovium has been shown to be richly innervated with Substance P immunoreactive nerve fibers, and synovial fluid has been shown to contain Substance P. However, the release characteristics of *spinal* Substance P, effected by internuncial spinal neurons, might offer the key to neural pain reception. To this end, a Substance P specific radioimmunoassay technique is being employed to quantify these neural substrates of perceived "pain" in the rat. Negative cannabinoidergic/ opioidergic modulation of Substance P release would represent suggestive evidence that these compounds are capable of producing a significant antinociception at the *transductive level* in a mammalian model of chronic pain and, therefore, would indicate further evidence as to the efficacy of the cannabinoids in attenuating human pain states.

LARGE DOSES OF THE CB_1 RECEPTOR ANTAGONIST SR141716A INCREASE SEROTONIN AND DOPAMINE LEVELS AND TURNOVER IN THE LEAST SHREW BRAIN

Nissar A. Darmani
Department of Pharmacology
Kirksville College of Osteopathic Medicine. Kirksville, MO 63501

Previously, we have shown that large doses of SR141716A (> 10 mg/kg, i.p.) induce vomiting in the least shrew (3). Depending upon the species used and the laboratories where observations were recorded, similar and smaller doses of SR141716A also produce the head-twitch response (HTR) and scratching behavior in drug naive animals (1, 2, 4, 5). The cited induced behaviors were potently blocked by diverse cannabinoid agonists (3, 4, 5). In addition, the selective 5-$HT_{2A/C}$ antagonist SR46349B, was shown to prevent SR141716A-induced HTR and scratchings in a potent and dose-dependent manner (4). These results suggest that large doses of SR141716A act as an inverse agonist and/or cause release of emetogenic neurotransmitters such as acetylcholine, dopamine or serotonin. In the present study, we investigated the effects of emetic doses of SR141716A on serotonin and dopamine levels and turnover in the shrew fore- and hindbrain. Thus, different groups of shrews were injected with either 0, 20 or 40 mg/kg SR141716A (i.p.) and their brains were rapidly removed and dissected for monoamine analysis at 15, 30, 60 and 120 minute posttreatment. Thus, brain supernatants were injected directly on to the column and were assayed for serotonin, dopamine and their major metabolites using the HPLC-ECD technique. Relative to the corresponding vehicle-treated control groups, these large doses of SR141716A caused dose- and time-dependent increases in forebrain serotonin (52-149%) and dopamine (60-101%) levels. The serotonin (5-HIAA) and dopamine (DOPAC and HVA) metabolites concentrations and the respective 5-HT (5-HIAA/5-HT) and dopamine (DOPAC/dopamine or HVA/dopamine) turnover were also increased in a similar fashion in shrew forebrain in response to SR141716A administration. Unlike the forebrain, concentrations of both 5-HT (29-48%) and dopamine (42-58%) were reduced in the shrew hindbrain. On the other hand, the levels of their corresponding metabolites as well as monoamine turnover tended to increase in the hindbrain. However, these enhancements were not as robust as those observed in shrew forebrain. Since previously it has been shown that lower doses of SR 141716A (<3 mg/kg, i.p.) has little effect on monoamine levels, the present data suggest that larger doses of SR141716A can alter functional activity of serotonergic and dopamine neurotransmitter systems. Release of one or both of the monoamine neurotransmitter may lead to the production of the observed emesis and serotonergic behaviors.

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THE CANNABINOID ANTAGONIST SR141716 INHIBITS FOOD INTAKE IN MICE BUT DOES NOT DECREASE ACTIVITY

Margaret A. Joppa, Stacy Markison, Kathleen R. Gogas, Alan C. Foster, and Greg S. Naeve
Neuroscience Department, Neurocrine Biosciences, Inc., 10555
Science Center Drive, San Diego, CA 92121-1102

The selective cannabinoid CB_1 receptor antagonist SR141716 is currently being evaluated in clinical trials for its therapeutic efficacy as a treatment for obesity. In rats, SR141716 was shown to reduce food intake, however few experiments have assessed the effects of this compound on feeding in mice. Analysis in this species is warranted given that food-intake in CB_1 knockout mice is not reduced by pretreatment with SR141716 but is significantly reduced in wild-type mice (C57BL/6J; Di Marzo et al, **2001**). The effects of SR141716 on food intake have been further studied to determine whether they are due to modulation of the rewarding value of food, direct effects on ingestive behaviors, or are dependent on high carbohydrate diets. Therefore, we endeavored to extend the above findings and determine the time course and dose-response of SR141716 on acute intake of sweet and standard chow in outbred mice. Female CD-1 mice (Charles River; n=10/group) were used for all experiments. Mice were housed 5 per cage with corncob bedding for at least 1 week before treatment. The animals were habituated to sweetened chow (Research Diets #C1110B, whole milk/sucrose diet) or standard chow (Purina 5053) presented in the bottom of their cages. Sixteen hours prior to the start of the experiment (2 hours before dark phase onset) mice were placed in a clean cage without food. On the day of the experiment, mice received injections [10 ml/kg, ip] of either SR141716 [1-30 mg/kg] or vehicle [0.1% Tween-80] and were placed in individual cages with water available. Thirty minutes after the injection, a pre-weighed pellet of food was placed in the cage. Food weight was measured 1, 2, 4, and 6 hours after food presentation in the standard chow experiment. The same time points were used for the sweetened chow experiment, with the addition of a 24-hour measurement. In a third experiment, we examined acute activity in addition to feeding behavior. SR141716 significantly reduced sweet chow consumption. This effect was significant at 1 hour for all doses and at 2, 4, and 6 hours for 10 and 30 mg/kg. There were no significant effects of SR141716 at 24 hours. SR141716 also reduced consumption of the standard chow, with a dose- and time-response similar to the sweetened chow. In vehicle-treated mice, intake of standard chow was not significantly different from intake of sweetened chow. Total, ambulatory, and stereotypic movements were not affected by any dose of SR141716 during the first or second hour of testing. These findings demonstrate that SR141716 inhibits consumption of both sweet and standard chow in hungry mice without altering general activity levels. Future experiments will address the effects of this compound on feeding and body weight with chronic administration.

SR141716 ATTENUATES HYPERPHAGIA INDUCED BY INTRA-PVN BUT NOT INTRA-ACCUMBENS MORPHINE

Aaron Verty, Malini Singh, Iain McGrego[1], and Paul Mallet
School of Psychology, University of New England, Armidale, NSW 2351 Australia
[1]Department of Psychology, Sydney University, Sydney, NSW 2006, Australia

A large body of literature suggests a role for the opioid system in mediating food intake. Despite numerous studies examining the stimulatory role of opioids in feeding, the specific mechanism of this action is yet to be clearly elucidated. One of the aims of the present study was to determine whether a cannabinoid receptor antagonist could attenuate the increase in food consumption produced by an opioid receptor agonist. In addition, the present study sought to identify specific brain regions underlying cannabinoid-opioid interactions in feeding behaviour. In Experiment 1, free-feeding male albino Wistar rats (N=12) were first injected with the CB_1 cannabinoid receptor antagonist SR141716 (0.03, 0.3, or 3.0 mg/kg or vehicle) intraperitoneally (i.p.) then allowed 30-min access to standard rat chow. Rats were then given vehicle or morphine HCl (2.5 mg/kg) subcutaneously and immediately reintroduced to the test environment. Each rat received one treatment every 48 hours in a counterbalanced order, during which food consumption was measured for 120 min. Results indicated that SR141716 significantly attenuated morphine induced food intake at the highest dose tested ($F_{3,47}$=10.51, p<0.001). In two subsequent experiments, free-feeding male albino Wistar rats were surgically implanted with bilateral guide cannulae aimed at the medial shell of the nucleus accumbens (Experiment 2, N=12) or a unilateral guide cannula aimed at the paraventricular nucleus of the hypothalamus (PVN, Experiment 3, N=14). Following a one-week recovery period, all rats were injected i.p. with SR141716 (0.03, 0.3, or 3.0 mg/kg or vehicle) and were then given 30-min access to standard rat chow. Rats were then injected with vehicle or morphine HCl (10 nmol in 1.0 µl) directly into the nucleus accumbens or PVN and then were reintroduced to the test apparatus. Food intake was assessed for 120 min. SR141716 significantly attenuated the hyperphagia produced by intra-PVN but not intra-accumbens morphine at all doses tested ($F_{4,69}$=14.61, p<0.001). Locomotor activity (time spent in motion) was assessed during the testing period in all experiments. Morphine significantly stimulated locomotion in all experiments, but this effect was not significantly attenuated by any dose of SR141716. Because SR141716 had no effect on hyperphagia produced the intra-accumbens application of morphine, it would appear that cannabinoid receptors do not modify the hedonic properties of food. Rather, we conclude that the suppression of morphine-induced hyperphagia produced by SR141716 may be due to an interaction with the opioid system within the hypothalamus, serving to suppress local satiety mechanisms.

DIFFERENTIAL MODULATION OF IMMEDIATE EARLY GENE EXPRESSION AND GABAERGIC INTERNEURON ACTIVITY IN THE CAUDATE PUTAMEN AND NUCLEUS ACCUMBENS IN RESPONSE TO THC

Alice Egerton[1], Judith Pratt[1], and Ros Brett[2]
[1]Department of Physiology and Pharmacology, Stathclyde Institute of Biomedical Sciences, University of Strathclyde, Glasgow, G4 0NR, UK [2] Division of Biological Sciences, University of Paisley, Paisley, PA1 2BE, UK

Δ^9-tetrahydrocannabinol (THC) produces biphasic dose effects upon reinforcement and locomotor activity. We hypothesised that these effects on behavioural output may be associated with differential patterns of activity in brain regions implicated in these behaviours. We therefore employed several complementary IEGs to map regional neural activation following acute administration of different doses of THC in the rat. In addition, parvalbumin mRNA expression was used as a marker of GABAergic interneuron activity. Rats were administered THC (0.01-5 mg/kg i.p.) or vehicle as control, and were euthanased 75 mins post-injection. The regional expression of mRNA encoding parvalbumin and the IEGs c-fos, fosB, c-jun, junB, zif-268 and nur77 was quantified using *in situ* hybridisation of radiolabelled oligonucleotide probes to coronal brain sections. Following subsequent densitometric analysis, data were analysed by ANOVA followed when appropriate by the Newman Keuls post-hoc test.

In the caudate putamen, THC induced uniphasic increases in fosB, junB, c-fos and nur77 following 1.0 and 5.0 mg/kg THC. In contrast, the pattern of expression with zif-268 appeared biphasic, with small decreases in expression at 0.01 mg/kg THC and increases in expression at the larger doses of THC with respect to 0.01 mg/kg but not to controls. In the nucleus accumbens, there were dose dependent increases in fosB and nur77 mRNA expression. However, increases in junB and zif-268 mRNA expression were more apparent after 1.0 mg/kg than 5 mg/kg in this region.

Expression of parvalbumin mRNA showed a contrasting pattern of regulation. In the caudate putamen and nucleus accumbens, this was a mirror image of the THC-induced increases in IEG expression. Thus there was a small increase in expression following 0.01 mg/kg THC whereas the larger dose (5.0 mg/kg THC) reduced expression compared to 0.01 mg/kg THC but not to controls.

In summary, these results suggest that the THC-induced increases in IEG expression in the caudate putamen were accompanied by reduced GABAergic interneuron activity. THC - induced alterations of IEG mRNA expression in the nucleus accumbens were more complex. This may reflect the dose dependent changes in activity of innervating structures (e.g. prefrontal cortex and hippocampus) which were also detected in this investig ation.

EVIDENCE FOR MOTOR DYSFUNCTION IN CB_1/CB_2 DOUBLE KNOCKOUT MICE

Meliha Karsak[1], Rustam Mundegar[2], Edda Erxlebe[1], Julia Essig[1], and Andreas Zimmer[1]
[1]Laboratory of Molecular Neurobiology, Department of Psychiatry,
University of Bonn, 53105 Bonn, Germany,
[2]Institute of Physiology II, University of Bonn, 53111 Bonn, Germany

In our lab the CB_1-/- (Zimmer *et al*., PNAS, **1999**, *96*, 5780) and CB_2-/- (Buckley *et al*., Eur. J. Pharm. **2000**, *396*, 141) receptor knockout mice were generated. While the disruption of the neuronally expressed CB_1 receptor resulted in readily detectable behavioral effects, there was no gross perturbation of immune functions in mice with a disruption of the peripherally expressed CB_2 receptor. To examine if there is a functional redundancy of both cannabinoid receptors, we generated CB_1-/- /CB_2-/- receptor double knockout mice.

The CB_1-/- /CB_2-/- animals are viable and fertile. They appear to be hypoactive like CB_1-/- mice, in addition they seem to have an abnormal gait. We therefore started to evaluate motor skills of these animals in behavioral experiments. Adult animals were tested in the rotarod and pawprint tests. For the rotating rod we used a constant speed for the first 20 sec and increased afterwards the speed uniformly. The experiments revealed significant differences between control animals and double knockout mice, and indicated that CB_1-/- /CB_2-/- animals have impaired motor functions. To determine whether a muscular weakness or a neuronal defect is the reason for the motor disabilities, we performed different histochemical analyses for fast and slow muscle fibers. Our results showed no differences between CB_1-/- /CB_2-/- and wild-type mice.

We can therefore exclude gross morphological changes in skeletal muscle which make a myogenic origin of the motor dysfunction unlikely, although functional disturbances of skeletal muscle have not been ruled out yet. There was also no evidence for muscular inflammation. Currently we are investigating a possible neurogenic origin of the motor phenotype.

ANANDAMIDE RELEASED FROM ESTROGEN-STIMULATED ENDOTHELIAL CELLS INHIBITS SEROTONIN SECRETION FROM PLATELETS

Mauro Maccarrone, Monica Bari, Natalia Battista, Marianna Di Rienzo,
Riccardo Pauselli, and Alessandro Finazzi-Agrò
Department of Experimental Medicine and Biochemical Sciences,
University of Rome Tor Vergata, I-00133 Rome, ITALY

Estrogen replacement therapy has been associated with reduction of cardiovascular events in postmenopausal women, though the mechanism for this benefit remains unclear. Here, we show that at physiological concentrations estrogen activates the anandamide membrane transporter of human endothelial cells (M. Maccarrone et al. *J. Biol. Chem*. 275:13484, **2000**), by binding to surface receptors and leading to rapid elevation of calcium (apparent within 5 min) and release of nitric oxide (within 15 min). We also show that binding of estrogen to the endothelial receptors leads to stimulation of the anandamide-synthetizing enzyme phospholipase D, and to inhibition of the anandamide-hydrolyzing enzyme fatty acid amide hydrolase, the latter effect being mediated by 15-lipoxygenase activity. Since the endothelial transporter is shown to move anandamide across cell membranes bidirectionally, taken together these data suggest that the physiological activity of estrogen is t o stimulate the release, rather than the uptake, of anandamide from endothelial cells. Moreover, we show that anandamide released from estrogen-stimulated endothelial cells, unlike estrogen itself, inhibits the secretion of serotonin, a pain signal, from ADP-stimulated platelets. Therefore, it is suggested that the peripheral actions of anandamide could be part of the molecular events responsible for the beneficial effects of estrogen.

ANANDAMIDE AFFECTS THE HYPOTHALAMO-PITUITARY-ADRENAL AXIS IN CB_1 RECEPTOR INACTIVATED MICE. A POSSIBLE PHYSIOLOGICAL BACKGROUND OF A NON CB_1 CENTRAL CANNABINOID RECEPTOR

G. Moldrich[1], C. Ledent[2], G. Tramu[3], and T. Wenger[1]
Department of Human Morphology and Developmental Biology, Semmelweis University, Budapest, P.O.Box 95, H-1450, Hungary (1), IRIBHN, Université Libre de Bruxelles, Brussels, Belgium (2)and Laboratoire de Neurocytochimie Fonctionnelle, Université Bordeaux I, Talence, France(3)

The endocannabinoid system (ES) takes part in several regulatory mechanisms of the central nervous system (CNS). The putative role of endocannabinoids in the regulation of hypothalamo-pituitary-adrenal (HPA) axis has been postulated. The effects of cannabinoids in the CNS is generaly attributed to their actions by the activation of central cannabinoid (CB_1) receptor. In rats it was already postulated that AEA activates the HPA axis via an unknown central cannabinoid receptor for which SR141716 is not an effective antagonist. Since transgenic mice laking gene encoding for CB_1 receptor represent a powerful mean to assess whether or not the inactivated receptor plays a role in a specific function the experiments were carried out in CB_1 receptor knockout mice.

Homozygote CB_1 +/+ and -/- mice were used. The animals were i.p. injected with one single dose of AEA (0,02mg/kg), AEA and SR141716 (30 prior AEA injection), SR141716 alone and vehicle, respectively. FOS protein expression (detected by immunohistochemistry) was used as index of cellular activation of the CNS.

45 min. after AEA administration in both +/+ and -/- mice a considerable number of nuclei in the parvocellular part of hypothalamic paraventricular nucleus (PVN) showed FOS immunoreactivity (FOSir). IF AEA treatment was preceeded by SR141716 no changes in FOSir were observed. SR141716 alone did not activate PVN.

AEA increased significantly the serum ACTH and corticosterone levels in both +/+ and -/- mice. SR141716 did not bloked the effects of AEA neither in FOSexpression nor in serum hormone levels. The results emphasize that there may exsits a, postulated but yet unknown, cannabinoid receptor of which activation is taking part in the regulation of HPA axis.

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NEUROPROTECTION OF RETINAL GANGLION CELLS UNDER GLAUCOMATOUS CONDITIONS

G. Ayoub, N. Garcia, L. Fischer, K. Roach, R. Chan,
A.Nudelman, A. Quirkm, and C. Morris
Department of Biology, Westmont College, Santa Barbara, CA 93108 USA
Neuroscience Research Institute, University of California,
Santa Barbara, CA 93106 USA

Introduction: Under glaucomatous conditions, excess levels of glutamate accrete in the retinal ganglion cell layer.

Methods: To test the putative neuroprotective effect mediated by cannabinoids, mice were given bilateral injections of 160 nmol NMDA to elicit glutamate induced retinal ganglion cell death. Under these conditions, 70% of the retinal ganglion cells undergo cell death within 10 days. Solutions containing the cannabinoid agonist WIN55,212-2 were injected intraocularly, or applied topically in a unilateral fashion.

Results: Following a ten day incubation period, animals were perfused and the retinae fixed and stained. Surviving retinal ganglion cells were counted and differential survival compared. The NMDA plus cannabinoid injected eyes retained two times the number of retinal ganglion cells as the eyes exposed only to NMDA.

Conclusion: It is proposed that cannabinoid agents may provide protection for glutamate-induced neurotoxicity under chronic or acute conditions.

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CANNABIDIOL PROTECTS AGAINST CEREBRAL-ISCHEMIA INDUCED EEG FLATTENING, HYPERMOTILITY AND COGNITIVE DEFICIT IN GERBILS

,Mariaelvina Sala, and Daniela Braida
Department of Pharmacology, Chemotherapy and medical Toxicology
University of Milan, Via Vanvitelli 32, 20129 Milan, Italy

Cannabidiol is a natural atypical cannabinoid, devoid of classical psychotropic effects of Δ^9-tetrahydrocannabinol and provided with a low toxicity (Rosenkrantz *et al., Toxicol. Appl. Pharm.* **1981**, *58*, 118; Mechoulam *et al*., *In: Marijuana/Cannabinoids: Neurobiology and Neurophysiology* **1992**) However, it produces some potentially therapeutic effects *in vitro* and *in vivo* assays. Neuroprotection against neurotoxicity mediated by NMDA, AMPA and kainate type receptors in rat cortical neuron cultures, exposed to toxic levels of glutamate, has been observed by cannabidiol (Skaper *et al*., *Proc. Natl. Acad. Sci.* **1996**, *93*, 3984; Twitchell *et al*., *J. Neurophysiol.* **1997**, *78*, 43; Hampson *et al., Proc. Natl. Acad. Sci.* **1998**, *95*, 8268). Through its combined immunosoppressive and antinflammatory actions, cannabidiol administered orally or i.p., has been shown to possess a potent anti-arthritic effects in a murine collagen-induced arthritis model (Malfait *et al*. *Proc. Natl. Acad. Sci.* **2000**, *97*, 9561).

In view of the these encouraging results and the recent findings of neuroprotective activity of the synthetic cannabinoids (WIN 55,212-2 and CP 55,940) against cerebral focal and global ischemia in rodents (Nagayama *et al. J. Neurosci.* **1999**, *19*, 2987; Braida *et al*. *Neurosci. Lett.* **2000**, *296*, 69), we established to assess a potential therapeutic activity of cannabidiol on transient global cerebral ischemia in the gerbil. The compound was injected i.p. in a wide range of doses (1.25 – 20 mg/kg.) 5 min after recirculation to verify a possible recovery against electroencephalographic (EEG) spectral power decrease, hyperlocomotion and cognitive deficit. Mean total and relative (from 0.2 to 25 Hz) spectral power was evaluated for 1 h before (basal) and 1 h, 1 and 7 days after ischemia. Spontaneous locomotor activity was evaluated at 1 and 7 days in an activity cage while memory function in a passive avoidance task 3 days after ischemia.

The dose-dependency showed a biphasic effect, with an optimal activity with 5-10 mg/kg in antagonizing the ischemia-induced reduction in mean total spectral power, tested 7 days after. Ischemia-induced hyperlocomotion, was significantly reduced at all tested doses 24 h after recirculation, in comparison with vehicle group. Ischemia-induced impairment of memory function was partially reversed, within 3 days, in the cannabidiol-treated group only with 5 mg/kg. Even if the mechanism of action has still to be elucidated, these findings suggest a potential therapeutic role of cannabidiol on cerebral ischemia.

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ACTIVATION OF THE CB_1 CANNABINOID RECEPTOR PROTECTS CULTURED SPINAL NEURONS AGAINST EXCITOTOXICITY

Mary E. Abood, Gulrukh Rizvi, Sonya Patel,
Sean D. McAllister, and Chandrasekaran Raman
Forbes Norris MDA/ALS Research Center, California Pacific Medical Center,
San Francisco, CA 94115 USA

Significant advances are being made towards understanding the genetic basis for amyotrophic lateral sclerosis (ALS) as well as the mechanistic and molecular pathways mediating progression of the disease; however, effective pharmacotherapy remains elusive. Two of the primary theories underlying motor neuron vulnerability are susceptibility to excitotoxicity and oxidative damage. Compounds that affect the anandamide/CB receptor system have the potential to reduce both excitotoxic and oxidative cell damage. This potentially synergistic action may lead to an improved therapeutic effect as compared to conventional glutamate antagonists or antioxidants. To evaluate the potential neuroprotective effects of cannabinoids in brain and spinal cord cultures, we determined whether Δ^9-THC could reduce excitotoxicity produced by direct application of excitatory amino acids. Primary cultures prepared from spinal cord were treated overnight in the presence of 100 μM kainic acid, 100 μM kainate plus 0.5 μM THC, 100 μM kainate plus 10 μM NBQX, 100 μM kainate plus 0.5 μM THC plus 1 μM SR141716A, or vehicle (control). Twenty-four hours later the cultures were evaluated for propidium iodide uptake as a visual measure of cytotoxicity. In conjunction with the visual quantification of cell death by propidium iodide uptake, an additional measure of cytotoxicity was employed, lactate dehydrogenase (LDH) release. The amount of cytotoxicity produced by 100 μM kainate (42%) was expected in a mixed spinal cord culture containing dorsal horn as well as motor neurons. We consistently observed attenuation of kainate toxicity by Δ^9-THC in these cultures. The extent of protection was comparable to that of NBQX, an AMPA/Kainate receptor antagonist. Our experiments indicated the effect of Δ^9-THC was mediated by the cannabinoid CB_1 receptor, as co-addition of the CB_1 receptor antagonist SR141716A blocked the protective effect of Δ^9-THC. However, the extent of protection by other exogenous and endogenous cannabinoids does not correlate with CB_1 receptor efficacy in other systems. Using an antibody directed against the N-terminus of CB_1, receptors were localized to spinal neurons and astrocytes by immunocytochemistry and Western analyses, respectively. Our data indicates for the first time evidence that the activation of the CB_1 cannabinoid receptor effectively modulates kainate toxicity in primary neuronal cultures prepared from mouse spinal cord.

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SUPPRESSION OF HUMAN MONOCYTE INTERLEUKIN-1β PRODUCTION BY AJULEMIC ACID (CT3), A NONPSYCHOACTIVE CANNABINOID

Bonnie Bidinger[1], Sumner H. Burstein[1,2],
Robert K. Furse[1], Ronald G. Rossetti[1], and Robert B. Zurier[1]
University of Massachusetts Medical School, [1]Department of Medicine and
[2]Department of Biochemistry and Molecular Pharmacology, Worcester, MA 01655

Oral administration of ajulemic acid (AjA), a cannabinoid acid devoid of psychoactivity, reduces joint tissue damage in rats with adjuvant arthritis (Zurier et al., **1998**). Because interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα) are central to progression of inflammation and joint tissue injury in patients with rheumatoid arthritis (RA), we investigated human monocyte IL-1β and TNFα responses after addition of AjA to cells in vitro. Peripheral blood and synovial fluid mononuclear cells (PBMC and SFMC) were isolated from healthy subjects and patients with inflammatory arthritis, treated with AjA (0-30 μM) in vitro, then stimulated with lipopolysaccharide (LPS). Cells were harvested for mRNA and supernatants were collected for cytokine assay. Addition of AjA in vitro to PBMC reduced both steady state levels of pro IL-1β mRNA and secretion of IL-1β in a dose dependent manner. Suppression was maximal at 10 μM AjA ($p<0.05$ vs untreated controls, n=6). AjA did not influence TNFα gene expression in or secretion from PBMC. Reduction of IL-1β by AjA may help explain the therapeutic effects of AjA in the animal model of arthritis. Development of therapeutically useful synthetic analogs of *Cannabis* constituents such as AjA may help resolve the ongoing debate about use of marijuana as medicine.

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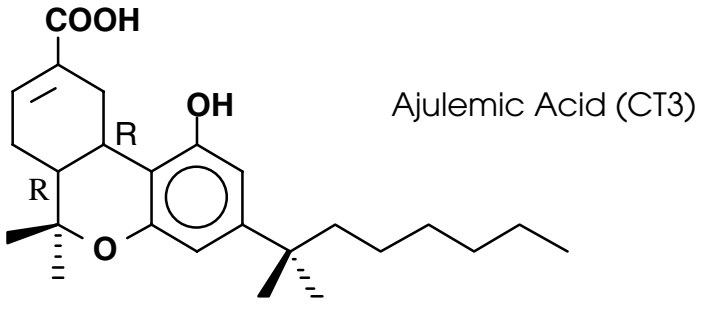

Ajulemic Acid (CT3)

THE CANNABINOID CB_1 RECEPTOR ANTAGONIST SR141716 INHIBITS CP55,940 BEHAVIOURAL EFFECTS AND REDUCES CASTOR-OIL DIARRHEA IN DOGS WITHOUT AFFECTING HASTENED GASTROINTESTINAL TRANSIT

Tiziano Croci[1], Luciano Manara[1], Massimiliano Bardotti[1],
Murielle Rinaldi-Carmona[2], Jean-Pierre Maffrand[3], and Gérard Le Fur[4]
[1]Research Center Sanofi Midy - Sanofi Synthelabo S.p.A., Via G.B. Piranesi 38, 20137 Milan, Italy,
[2]Sanofi Synthelabo, 371 rue du Pr. J. Blayac, 34184 Montpellier, France,
[3] Sanofi Synthelabo, 195 route d'Espagne, 31036 Toulouse, France,
[4] Sanofi Synthelabo, 174 avenue de France, 75635 Paris, France

The effect of the selective cannabinoid CB_1 receptor antagonist, SR141716 (N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide) on diarrhea and hasting of gastrointestinal transit by castor oil was compared with that of the non selective cannabinoid receptor agonist, CP 55,940, the selective CB_2 antagonist SR144528 (N-(1S)-1,3,3-trimethylbicyclo(2.2.1)-hept-2-endo-yl)-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide) and loperamide. Seven male beagle dogs (10 ± 2 kg) were given castor-oil or corn-oil intragastrically (1 ml/kg) 2 h after a light meal (120 g of a commercial moist food at 8.00 AM) containing 40 non absorbable soft plastic markers (1.5 Ø, 4 mm length). Drugs were administered with the meal (po) or at same time of castor-oil (sc). The markers expelled with the feces were collected and counted 8 and 24 h from administration. Behaviour, diarrheal and emetic events were monitored for 24 h. Two weeks washout period was allowed between two treatments.

Table 1.

		CASTOR-OIL			CORN-OIL		
		dogs with diarrhea	% markers' output[a] *8 h*	*24 h*	dogs with diarrhea	% markers' output[a] *8 h*	*24 h*
Vehicle		5/5	32 ± 15	66 ± 12	0/5	1 ± 1	65 ± 3
Loperamide	0.05 mg/kg, po	5/5	8 ± 5*	34 ± 14	0/5	11 ± 8	55 ± 11
	0.15	2/5	7 ± 6*	24 ± 3*	0/5	3 ± 2	37 ± 17
CP 55,940	0.01 mg/kg, sc	4/5[b]	***ne***	***ne***	0/3[b]	0 ± 0	37 ± 19
	0.1	1/5[c]	10 ± 8*	70 ± 13	0/5[bc]	0 ± 0	65 ± 9
SR141716	1 mg/kg, po	3/5	13 ± 6	35 ± 14	0/5	19 ± 9*	72 ± 16
	3	1/5	37 ± 9	84 ± 3	0/5	5 ± 2	63 ± 6
SR144528	3 mg/kg, po	4/5	16 ± 3	58 ± 3	0/3	8 ± 3	73 ± 13

[a] Figures are mean ± SE. * $p < 0.05$, vs vehicle (Duncan's test). [b] The dogs vomited 2-4 h after the drug. [c] All dogs lay prostrate on the floor and showed marked ataxia. ***ne*** not evaluable, because of vomited markers.

As shown in Table 1, castor-oil induced diarrhea and increased markers' output. SR141716 (1-3 mg/kg, po), unlike SR144528, dose-dependently inhibited castor-oil diarrhea, but had no effect on markers' output in either corn oil or castor oil treated dogs. CP 55,940 (0.1 mg/kg, sc) prevented diarrhea, but also induced long lasting (8-12 h) behavioural effects that were prevented by SR141716 (3 mg/kg, po). CP 55,940 (0.01 mg/kg, sc) and loperamide had no behavioural effects, however the former induced emesis. Loperamide delayed hastened markers' transit and, only at the higher dose, prevented diarrhea. The present findings attest the potential antisecretory-antidiarrheal activity of the cannabinoid CB_1 receptor antagonist SR141716 that, unlike loperamide, had no significant effect on hastened markers' transit.

UCM707, A POTENT AND SELECTIVE INHIBITOR OF THE ENDOCANNABINOID UPTAKE, POTENTIATES HYPOKINETIC AND ANTINOCICEPTIVE EFFECTS OF ANANDAMIDE

Eva de Lago, Javier Fernández-Ruiz, Silvia Ortega-Gutiérrez[1], Alma Viso[1], María Luz López-Rodríguez[*], and José A. Ramos
Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Complutense, 28040-Madrid, Spain; [1]Departamento de Química Orgánica, Facultad de Ciencias Químicas, Universidad Complutense, 28040-Madrid, Spain

To date, UCM707, N-(3-furylmethyl)eicosa-5,8,11,14-tetraenamide, is the inhibitor of the endocannabinoid transporter with the highest potency and selectivity *in vitro*, which might enable this compound to be used to potentiate the endocannabinoid transmission, with minimal side-effects, in the treatment of several disorders. However, there is no information about how UCM707 behaves *in vivo* as regards to some classic effects of endocannabinoids, as hypomotility and antinociception. In the present work, we tested in rats the dose-response effects of UCM707 in the open-field test and hot-plate, and, in particular, we analyzed if this compound was able to enhance the hypokinetic and/or the antinociceptive actions of anandamide, at a subeffective dose, using these two *in vivo* assays. UCM707, administered alone, had negligible effects on ambulatory, exploratory and stereotypic activities, time spent in inactivity and sensitivity to pain, with only some small responses at the highest dose used. UCM707, administered at a dose that did not produce any effects by itself, was, however, able to significantly potentiate the action of a dose of anandamide that had not produced any effects when it was administered alone. So, the combination of both compounds produced higher decreases in the exploratory activity and, particularly in ambulation, and increased the time spent in inactivity and the latency to respond to a painful stimuli. In summary, UCM707, as suggests its *in vitro* properties, also behaves *in vivo* as a selective and potent inhibitor of the endocannabinoid transporter showing negligible direct effects on the receptors for endocannabinoids but potentiating the action of these endogenous compounds. This allows to consider this compound as a promising tool, used alone or in combination with endocannabinoids, for the treatment of a variety of disorders where a hypofunction of the endocannabinoid transmission has been postulated.

CBD AND THC EXTRACTS ALTER BEHAVIORAL DESPAIR ON THE MOUSE TAIL SUSPENSION TEST

Richard E. Musty[1], Melissa J. Bouldin[2], Jacey R. Erickson[2], and Richard A. Deyo[2]
[1]Department of Psychology, University of Vermont, Burlington, Vermont 05405, USA and [2]Department of Psychology, Winona State University, Winona, Minnesota 55987, USA

Several investigators have speculated that cannabis use has been used for self-medication of depression (Degenhardt & Lynskey, Soc. Psychiatry. Psychiatr. Epidemiol., 2000, 36, 219; Diego & Sanders, Adolescence, 2001, 36, 491; Tunving, ACTA Psychiatr. Scand., 1985, 72, 209). To test this hypothesis, cannabis extracts containing cannabidiol (CBD) or Δ^9-tetrahydrocannabinol (THC) were tested for antidepressant effects.

In the first experiment, 112 C57BL6BYJ mice were weighed and randomly assigned to groups with the restriction that equal numbers of males and females be represented in each group. These groups were 0, 0.5, 1, 2, 4 or 8 mg/kg/0.01 cc/g body weight of THC extract (supplied by GW Pharmaceuticals, Porton Down, UK) or 30 mg/kg/0.01 cc/g body weight of imipramine. The mouse tail suspension test of behavioral despair was used because of evidence that it is a valid model of human depression. Attempts to escape by struggling are effectively reversed with antidepressants currently approved for human use (Steru et al., Psychopharm., 1985, 85, 367; Vaugeois et al., Psychopharm., 1997, 134, 387). All drugs were injected intraperitoneally 30 minutes before testing on the tail-suspension test. Each mouse was suspended from a bar by the tail using adhesive tape. The total number of movements and total amount of time spent immobile were recorded by a microcomputer via a photodiode connected to a computer interface placed 5 cm away from the subject and aimed at the middle of the subject's ventral surface. Each subject was tested for six minutes. Immediately after the tail suspension test, each mouse was individually placed in the open-field chamber for five minutes. Activity, rearing, grooming and defecation rates were recorded.

For THC, significant drug effects were found on both the number of movements ($F(6,98)= 7.494$, $p < .0001$) and the amount of time spent immobile ($F(6,98)= 8.327$, $p < .0001$) on the tail suspension test. Post-hoc analyses using Dunnett's test for comparisons involving a control mean (Dunnett, 1955; Kirk, 1982) showed that animals treated with imipramine (30 mg) made more movements (mean = 824.875, SD=479.414) and spent less time immobile (mean = 135.212s, SD=91.199) than controls (mean number of movements = 572.625, SD=425.056; mean time immobile = 194.106s, SD=70.460). THC at dosages of 4 mg and 8 mg produced significant reductions in the number of movements and significant increases in the amount of time spent immobile (4 mg mean time immobile=273.8 s, SD= 112.9; 8 mg mean time immobile=268.3 s, SD= 58.6) relative to vehicle treated controls (mean time= 194.106s, SD=70.460). There were no significant differences between controls and any other dose for either measure of behavioral despair.

For CBD, procedures were identical to those in the THC experiment except that drug groups were 0, 5, 10, 20, 40 or 80 mg/kg/0.01 cc/g body weight of a CBD extract (also supplied by GW Pharmaceuticals) or 30 mg/kg/0.01 cc/g body weight of imipramine.

Unlike THC, CBD produced behaviors that were consistent with imipramine on the tail suspension test (i.e., elevated activity: 20 mg CBD dose mean= 985.9, SD=404.9; control mean= 550.3, SD=467.2; imipramine 1171.5, SD 623.2). There was also a significant gender x drug interaction. Analysis of the interaction indicated that females required a higher dose of CBD than males before showing the antidepressant effect. However, the highest CBD doses (40-80 mg) reduced activity and increased immobility (similar to THC). These data suggest that CBD (and not THC) may represent an antidepressant component in cannabis.

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AN INHIBITORY ROLE FOR CANNABINOIDS IN THE CONTROL OF MOTION SICKNESS IN *SUNCUS MURINUS*

Farideh A. Javid[1], Colin Wright[1], Robert J. Naylor[1],
Brian A. Whittle[2], and Geoffrey W. Guy[2]
[1]School of Pharmacy, University of Bradford, Bradford, BD7 1DP, UK
[2]Porton Down Science Park, Salisbury, Wiltshire, Sp4 0JQ, UK

Motion sickness is generally attributed to a mismatch of sensory information from the vestibular, proprioceptive and the visual systems (Yates *et al*., **1990**). The available anti-motion sickness drugs such as muscarinic acid and histamine H1 antagonists do not provide a complete protection in all the subjects and therefore more effective treatments are required. Recent studies have shown that cannabinoids are useful in treating nausea and vomiting associated with anaesthesia after abdominal surgery (Lewis *et al*., **1994**), as well as radiation therapy (Priestman *et al*., **1987**). In previous studies, we have shown that an emetic response can be reliably induced in *Suncus murinus* (Javid and Naylor, **1999**). The aim of the present study was to investigate the effect of cannabinoids in motion sickness.

Two extracts of cannabis were used. High THC extract, (M6), contained a total of 51.4% of THC and THCA, 0.9% of CBDA and 1% CBN. M16 the high cannabidiol extract contained a total of 56.0% CBD and CBDA, and 1.4% THC. Adult female Japanese House Musk shrew, *Suncus murinus,* (32-42 g) were injected with either the M6 or M16 extract (1.0, 2.0 or 4.0 mg/kg, i.p.), or vehicle 30 min prior to a motion stimulus (1Hz, 40mm amplitude of shaking for 10 min) and observed for any overt behavioural change. The number of emetic episodes and the latency of onset(s) were recorded. Data were expressed as the mean ± s.e.m. of n=6 and analysed using ANOVA which was followed by Bonferronni-Dunnett's t-test. The administration of M16 at 1.0 and 2.0 mg/kg, but not 4.0 mg/kg, significantly ($p<0.05$) reduced the number of emetic episodes from 12.8±2.4 emetic episodes in vehicle treated animals to 6.3±1.4 and 6.16±2.4 respectively; this was followed by a significant ($p<0.05$) increase in the latency of onset of the first emetic episode from 103.0±25.3 seconds in the vehicle treated animals to 206.8±33.9 and 250.5±84.5 seconds, respectively. However, the administration of M6 at 1.0 and 2. mg/kg did not modify motion sickness. Indeed, M6 at 4.0 mg/kg significantly ($p>0.05$) intensified motion sickness by approximately 50%, and that was followed by a shorter onset of response as compared to the vehicle treated animals. None of the extracts were emetic in their own right and there were no other overt changes in behaviour of the animals. These data suggest that cannabinoids play a role in mediating motion sickness and that cannabidiol is likely to have an anti-motion sickness effect.

Data are presented on the relative contribution made by the constituents of these extracts to the anti-emetic effect.

CHRONIC TREATMENT WITH Δ^9-TETRAHYROCANNABINOL REDUCES DOPAMINERGIC INJURY IN AN EXPERIMENTAL MODEL OF PARKINSON'S DISEASE

Isabel Lastres-Becker, María Gómez, Rosario de Miguel, Ana Cabranes, Mariluz Hernández, Maribel Cebeira, José A. Ramos, and Javier Fernández-Ruiz
Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Complutense, 28040-Madrid, Spain

Recent data have suggested that plant-derived, synthetic or endogenous cannabinoids might be neuroprotectant in a variety of *in vivo* and *in vitro* models of neuronal injury. However, the mechanisms involved in these effects seem to be diverse, including from events not mediated by CB_1 receptors (NMDA antagonism, antioxidant properties) up to CB_1 receptor-mediated phenomena (inhibition of glutamate release or stimulation of GABA action). Parkinson's disease (PD) is a neurodegenerative motor disorder where an overactivity of the endocannabinoid transmission has been recently demonstrated. Thus, increased CB_1 receptor binding and mRNA expression have been revealed in the basal ganglia of humans with PD and MPTP-treated marmosets (Lastres-Becker et al., Eur. J. Neurosci. 14, 1827-32, **2001**), as well as in rats with unilateral lesions of the nigrostriatal dopaminergic neurons caused by 6-hydroxydopamine (Romero et al., Life Sci. 66, 485-494, **2000**), whereas higher endocannabinoid contents were measured in reserpinized rats (Di Marzo et al., FASEB J. 14, 1432-38, **2000**). These data have attracted interest to the possibility that the blockade of CB_1 receptors might be effective for the treatment of the signs of motor deterioration in this disease, although the data have not been conclusive. Here, we addressed for the first time whether cannabinoids, rather than altering the motor signs of this disease, might behave as neuroprotectant substances. To this end, Sprague-Dawley rats were subjected to unilateral injections of 6-hydroxydopamine into the medial forebrain bundle and, 16 hours later, they were subjected to a daily treatment with Δ^9-tetrahydrocannabinol (Δ^9-THC, 3 mg/kg) or vehicle during a period of 13 days. Two hours after the last injection, animals were sacrificed. Results showed that, as expected, the unilateral injection of 6-hydroxydopamine reduced (approx. 50%) the contents of dopamine (DA) and its metabolites and the activity of tyrosine hydroxylase (TH) in the caudate-putamen of the lesioned side. This reduction was accompanied by a decrease of TH-mRNA levels in the substantia nigra. However, the treatment with Δ^9-THC produced a recovery in most of these parameters compatible with the notion of a delay in the progression of the neuronal injury caused by the toxin. Taken together, the present data suggest a possible beneficial effect of cannabinoids in the treatment of PD, a motor disorder, which has a useful symptomatic treatment, but, as other neurodegenerative diseases, lacks of an efficient neuroprotectant therapy.

Δ^9-THC MODULATES THE ACUTE EFFECTS AND THE LONG TERM NEUROTOXICITY OF MDMA ("ECSTASY") IN RATS

Iain McGregor, Kirsten Morley, Kong Li, Kelly Clemens,
Helen Duffield, Paul Mallet[1], June Young[1], and Glenn Hunt
School of Psychology, University of Sydney, NSW 2006, Australia, and
[1]School of Psychology, University of New England, Armidale NSW 2351, Australia

MDMA ("Ecstasy") is a drug that is widely used by young people in many countries. There is increasing evidence that heavy use of the drug damages brain 5-HT systems and that this could lead to chronic emotional and cognitive problems. We have recently shown that male Wistar rats briefly exposed to MDMA (4 x 5 mg/kg i.p. over four hours on each of two days) show long term increases in anxiety-like behavior when assessed in the drug-free state three months after MDMA (Morley et al EJP (**2001**) 433, 91-99). Human MDMA users frequently consume cannabis while under the influence of MDMA and in ongoing work we have examined whether this might modulate the long-term behavioural and neurotoxic effects of MDMA in rats. The active constituent of cannabis Δ^9-THC (4 x 2.5 mg/kg over 4 h) reversed the acute hyperthermic effects of MDMA (4 x 5 mg/kg) in rats on each of two days of administration, with rats given the MDMA/THC combination showing a robust hypothermia. One month later rats that had received the MDMA/THC combination were found to be marginally less anxious on the emergence test and social interaction tests than rats that had previously received MDMA alone. Neurochemical analysis (HPLC) at six weeks post drug showed that Δ^9-THC attenuated the 5-HT depletion produced by MDMA in the hippocampus, striatum, amygdala and cortex. Immunohistochemistry using a 5-HT antibody allowed visualization of extensive 5-HT axonal degeneration in rats given MDMA relative to control rats. Again, Δ^9-THC provided partial protection against this effect. We hypothesise that by minimising the acute hyperthermic effects of MDMA, Δ^9-THC may partly protect against MDMA neurotoxicity and associated long term changes in mood and behaviour.

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CANNABIS SUBSTITUTION: HARM REDUCTION TREATMENT FOR ALCOHOLISM AND DRUG DEPENDENCE

Tod H. Mikuriya, and Jerry Mandel
California Cannabis Research Medical Group, Berkeley, CA 94708-1757, USA

Introduction:
Cannabis was first described by Clendinning in 1843 to treat withdrawal symptoms of alcoholism and opiate dependence. Delirium tremens was an indication in materia medica and withdrawal from opiates and choral hydrate described by Birch **1889**. Ethnographic studies have compared cannabis with alcohol use (Indian Hemp Drugs Commission **1894**, Carstairs **1954**) In 1970 the author reported a single case of cannabis substitution as maintenance to prevent alcoholism relapse.

Method:
The author interviewed and examined patients seeking a physician statement of recommendation and approval of medical marijuana under the California Compassionate Use Act of **1996**. Annual follow up interviews were conducted. Specific questions were centered around drinking behavior, use of prescription, non-medical drugs, and concurrent lifestyle changes. Recommendation was made to each patient to discontinue drinking and stop associating with other fellow users where possible. Review of 104 cases of the total of 6500 in the author's where ICD-9 CM 303.0 (Alcoholism), 304.0 (opioid dependence), 304.1 (sedative dependence), 304.2 (cocainism) 304.4 (amphetamine dependence), and 305.0 (alcohol abuse) were tabulated.

Results:
1. All of the persons interviewed had chronic alcoholism, opioid, stimulant, or sedative dependence.
2. Patients were frequently from a family where one or both parents were alcohol abusers or mentally ill (62%)
4. Alcoholism was the most frequent condition (90%)
5. Injuries when using alcohol (50%) add to the use of other drugs.
6. Intercurrent njuries with chronic pain or other degenerative conditions were frequent (60%)
6. All reported benefits from substituting cannabis.(100%)
7. Concurrent depression was significantly improved.(100%)

Conclusions:
Cannabis substitution for self selected alcoholics, opiate, sedative and stimulant dependency is salutary for its effects both controlling craving for more harmful dependence producing drugs. The efficacy of cannabis substitution first described by the author of one case treated in **1970** is substantially confirmed by independent "N of 1" reports in the treatment of alcoholism and other drug dependence.

CLINICAL REPORT: MEDICAL USES OF CANNABIS IN CALIFORNIA 2002

Tod H. Mikuriya, William Eidelman, Steven Ellis, Phillip Denney,
Marion Fry, George Fuller, Richard Hansen, Jeff Hergenrather,
Frank Lucido, Thomas O'Connell, and Eugene Schoenfeld
California Cannabis Research Medical Group, Berkeley, CA 94708-1757, USA

Introduction:
The Compassionate Use Act of 1996 (Health & Safety Code section 11362.5) exempts patients from prosecution from California marijuana laws. Physicians who recommend or approve are protected: "No physician in this state shall be punished, or denied any right or privilege, for having recommended marijuana to a patient for medical purposes" (C)2(c). Method: The authors interviewed and examined patients seeking a physician statement of recommendation and approval.

Results:

1. All of the persons interviewed had chronic medical conditions.
2. The most salient finding is the safety and freedom of undesirable effects of cannabis compared with conventional pharmacotherapy.
3. The toxicity of criminality adversely affects individual's self perception and well-being.
4. Cannabis is harm reducing through substitution for more toxic medical and social drugs.
5. Immunomodulation analgesia effective in posttraumatic sequellae and autoimmune or idiopathic inflammatory conditions.
6. Antispasmodic and antispastic for striated and smooth muscle, ant iseizure drug.
7. Mood modulator, antidepressant and cognition enhancer.

Clinical Pharmacologic:
Peripheral sites of action appear to be involved in varying degrees. Physical effects may preceed psychologic subjective changes using inhaled cannabis especially in spasticity. Further biomonitoring is needed to explore mechanisms of action.

Conclusions:
Observations by clinicians and pharmaceutical indications listed prior to 1937 are substantially confirmed our independent "N of 1" reports. Conditions reported using International Classification of Diseases elaborates and extends the range of therapeutic applications for which cannabis may be useful. Removal of criminal stigma is a positive psychotherapeutic intervention.

MEDICINAL CANNABIS EXTRACT IN CHRONIC PAIN: DESIGN OF A COMPARATIVE "N OF 1" PRIMARY STUDY (CBME-1)

William Notcutt, Mario Price, Samantha Newport, Cathy Sansom,
Sue Simmons, and Cheryl Phillips
James Paget Hospital, Lowestoft Road, GREAT YARMOUTH, Norfolk, NR31 6LA, UK

Introduction

Plant Cannabis is widely used by patients suffering with chronic pain. Previous attempts to study its use have been complicated by the inability to standardise the cannabis extract and to measure the amount of cannabinoid used. We report the methodology of a study to investigate the use of Cannabis Based Medicinal Extracts (CBME) in patients with chronic pain. This study is the first to use a metered sublingual dose spray containing known quantities of pharmaceutically prepared Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) extracted from plants grown under controlled conditions. The aims of the study were to determine the relative therapeutic windows, and to evaluate benefits and safety.

Methods

An "N of 1" format was used for the study because of the heterogeneous nature of chronic pain conditions. The medicinal cannabis extract was administered as a sublingual spray. Initially the patients underwent a two-week open run-in period (Part 1) using a 1:1 mixture of cannabidiol and Δ^9 tetrahydrocannabinol (THC/CBD). If they gained benefit from this they then progressed to Part 2 of the study, which included two blocks of four weeks. During this time each patient received one week of each of high THC, high CBD, placebo and 1:1 THC/CBD. The order of the CBME was randomised and double blind. Patients who were regular cannabis users were given rescue medication of the THC/CBD to prevent them returning to their previous cannabis use. Patients attended weekly for a variety of assessments (Pain and Activity scores, General Health Questionnaire (GHQ28), Becks Depression Inventory (BDI). Following these assessments, the medication for the subsequent week was then titrated to an appropriate level. VAS pain scores were recorded and samples for serum concentrations of THC and CBD were taken during the titrations. Non-invasive BP, pulse and ECG were monitored. Throughout 12 weeks, the patients kept a daily diary (VAS pain, sleep, side-effects, effectiveness, medication used). Those who gained benefit from the use of the cannabis extract were considered for entry into a long-term safety study.

Results

Patients with Multiple Sclerosis, chronic back pain & sciatica after spinal surgery and other neuropathic pains have been recruited. The first 6 patients (5 regular "medicinal" cannabis users, 1 previous "medicinal" cannabis exposure) gave early information on safety and effectiveness of the new materials. 34 patients have been studied and a wide variety of benefits have been observed.

Discussion

This method of studying a "new" drug and a new route of administration proved to be successful. It identified benefits and problems in using CBME and facilitated the design of subsequent trials which are now starting. In addition This method is close to the way in which a clinician would normally practice. More detailed results are reported in other posters and abstracts for this meeting.

MEDICINAL CANNABIS EXTRACT IN CHRONIC PAIN: RESULTS FROM LONG-TERM SAFETY EXTENSION STUDY (CBME-SAFEX)

William Notcutt, Mario Price, Samantha Newport, Cathy Sansom, and Sue Simmons
James Paget Hospital, Lowestoft Road, GREAT YARMOUTH, Norfolk, NR31 6LA, UK

Introduction:
Currently the main target for the use of cannabinoids is in the management of chronic disease. Therefore the study of these drugs over months and even years is essential. The control of symptoms, the changes in drug use, activity & quality of life and the incidence of side effects are the critical issues. This study follows on from our primary CBME-1 study to assess the effectiveness of sublingual Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) (see Abstract 1,2 from this team)

Methods:
Patients with chronic pain from Multiple Sclerosis, spinal pain (post spinal surgery) and a variety of other neurogenic pains had completed the CBME1 study (see Poster 2) were recruited. All had shown benefit from either THC and/or THC:CBD mixture. For the first 8 months of the study, the Medicines Control Agency would only allow the use of THC. Later CBD was allowed. Patients individualised their CBME usage under guidance from the team. The same monitoring tools that had been used in the initial study were continued, though simplified. Patients were counselled about driving.

Results:
24 patients have been studied for between 2 and 19 months (following the initial 10 week study). Most have shown preference for the THC:CBD 1:1 mixture.

Symptom Control:
All patients initially maintained symptom control, although several took time settling onto the new regime. The previous routine of a different cannabinoid each week had been difficult and unsettling for some. In using a single cannabinoid continually they were able to customise their use to achieve optimal benefit. Many were also able to develop their activities.

Cannabinoid And Other Drug Use:
Most patients have maintained a static level of cannabinoid use. Some have managed to slowly reduce other medication but this has not been dramatic. Others have reduced their cannabinoid usage over time. None have significantly increased their intake. Several patients have stopped their cannabinoid medication for periods of time up to 1 month. None have reported any withdrawal symptoms but most have experienced an increase in pain on doing so.

Side Effects:
No major side-effects have emerged over the duration of our study.

Other Problems:
During this study many patients have encountered a range of physical and psychosocial problems (Life Events). These include exacerbation of MS (3), marital difficulties, conception and birth of a baby, injuries, death of a spouse (2) etc. All may substantially influence symptom control, mood and quality of life making evaluation of the benefits of CBME more complex. 2 patients have continued to experience significant problems with their pain management.

Discussion:
Sublingual Cannabis Medicine has been shown to provide continuing benefit for a group of patients with intractable pain. However, larger numbers of patients studied over longer periods of time will be needed to increase confidence in the long-term effectiveness and safety of cannabinoid extracts.

ENDOCANNABINOID GENETICS

Emmanuel. S. Onaivi[1, 2], Hiroki Ishiguro, Pingwu Zang[2], Babatunde. E. Akinshola[3] Zhicheng Lin[2], Nissar Darmani[4], Lumir Hanus[5], Bruce Hope[6], and George R. Uhl[2]
[1]William Paterson University, Wayne, NJ 07470, [2]Molecular Neurobiology Branch, NIDA Intramural Program, National Institutes of Health, Baltimore, MD 21224, [3]Howard University, College of Medicine, Washington, DC, 20059., [4]Kirksville College of Osteopathic Medicine, Kirksville, MO 63501, [5]Hebrew University, Jerusalem, Israel 91120., Behavioral Neuroscience Branch, NIDA Intramural Program, National Institutes of Health, Baltimore, MD 21224

Significant progress has been achieved in marijuana (cannabinoid) research. However little information is available at the molecular level about the polymorphic nature of the cannabinoid receptor (*Cnr*) gene structure, regulation and function. We have continued studies on *Cnr* genetics and signaling in order to understand the role of endocannabinoids in human behavior and physiology. Using in-vivo and in-vitro techniques we present data on human *Cnr* CB_1 gene transcript size, splice variants, SNPs, trinucleotide repeat and linkage disequilibrium between these polymorphisms in selected ethnic populations. While the frequencies of the SNPs differed by race, there was no significant linkage disequilibrium between the polymorphisms in three populations. Differences were observed when the effects of the endocannabinoids, anandamide, 2-AG and noladin ether were compared on kainate activated AMPA receptor subunits in *Xenopus* oocytes using the two-electrode voltage clamp. The presence of *Cnr* CB_1 protein and gene expression in the shrew brain and gut, mouse and rat brains and in human blood is presented as further evidence for the existence of an endocannabinoid physiological control system (EPCS). In addition the effects of *Cnr* agonists and the CB_1 antagonist, SR141716 in the rodent model of withdrawal aversions from cocaine, alcohol and diazepam support a role for this EPCS in natural reward regulatory mechanism. Therefore, understanding the EPCS in the human body and brain will contribute to elucidating this natural regulatory mechanism in health and disease.

NON-CB_1 RECEPTOR-MEDIATED CONTROL OF SPASTICITY

Gareth Pryce[1], Deborah R.J. Hankey[1], Peter Brown[1], Cristina Visintin[1], Catherine Ledent[2], Tiziana Bisogno[3], Raj K. Razdan[4], Billy R. Martin[5], Andrew S. Rice[6], Vincenzo Di Marzo[3] and David Baker[1]
[1]Institute of Neurology, University College London, WC1N 1PJ, UK, [2]IRIBHN, Free University of Brussels, B-1070, Belgium [3]Istituto di Chimica Biomolecolare, 80078 Pozzuoli, Italy, [4]Organix, Inc., Woburn, MA 01801, [5]Virginia Commonwealth University, Richmond, 23298, USA, [6]Imperial College, London SW10 9NH, UK

The cannabinoid system mediates tonic control of spasticity in the ABH mouse model (EAE) of multiple sclerosis (MS. Baker *et al. Nature*, **2000** 404:84, 2000. *FASEB.J* **2001**;15:300). Whilst CB_1 agonists mediate the major anti-spastic effect, symptom modification was observed with CB_2-selective compounds (Baker *et al.* 2000). However as such compounds are selective, rather than specific, agonists, demonstration of activity independent of CB_1, may best be shown by inhibition of spasticity in CB_1 gene (*Cnr1*) knockout mice. Whilst there is increasing evidence that CB agonists can inhibit painful spasms in multiple sclerosis (MS), local application of VR-1 agonists inhibit bladder hyper-activity in MS. Therefore a hybrid molecule that stimulates the cannabinoid and vanilloid systems may target multiple symptoms that occur in MS. Arvanil binds all of these receptor systems and is a potent analgesic agent. The anti-spastic potential was investigated. Experimental allergic encephalomyelitis (EAE) was induced by injection of mouse myelin antigens in Freund's adjuvant and after repeated paralytic episodes mice developed spasticity (within 60-100 days Baker *et a.l* 2000). Spasticity was assessed by resistance to flexion of hindlimbs against a strain gauge, prior and following drug administration. (Baker *et al*. **2000**). Congenic ABH.*Cnr1* -/- were generated by backcrossing ABH mice with CD1.*Cnr1*-/- knockout mice. (Ledent et al. *Science* **1999**. 283:401). Arvanil (0.1-0.01mg/kg i.v.) was a potent inhibitor of spasticity at doses where a CB_1 agonist (methanandamide) was inactive. Surprisingly high-dose VR-1 agonists (capsaicin at 0.1mg/kg i.v.) induced a minimal (10-15% compared to 35-50% inhibition of spasticity of cannabinoid agonists) anti-spastic response that could be inhibited by capsazepine (40kg/kg iv.) but also by CB_1/CB_2 antagonists (SR141617A + SR144528. 5mg/kg i.v.). In contrast the anti-spastic effect of arvanil was essentially unaffected by pretreatment with CB_1, CB_2 and VR-1 antagonism, and was also evident in CB_1-deficient mice. Although CB_2 agonists dose-dependently inhibited spasticity, this is probably due to cross-reactivity to CB_1. This is demonstrated by the lack of efficacy of CP55,940 (CB_1/CB_2 agonist) in spastic CB_1-deficient mice. This definitively indicates the activity of arvanil was independent of CB_1/CB_2. However even in *Cnr1* -/- mice arvanil still exhibited "tetrad" effects. An Arvanil-analogue 0-2093 (Di Marzo et al, *J Pharm Exp Ther* **2002**. 300:984), which is likewise potent in "tetrad tests", but has low affinity for CB_1 and CB_2 and essentially does not bind to VR-1, was similarly a potent anti-spastic agent. This suggests that there are other, as yet unknown, receptors that mediate therapeutic cannabinoid-like effects.

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MEDICAL GRADE CANNABIS© (MGC©), A PRODUCT OF MARIPHARM

L. Saddik[1], M. de Wit[1], and W.G. van der Sluis[2]
[1]Maripharm, Nieuw Mathenesserstraat 33, NL-3029 AV Rotterdam
[2]Universiteit Utrecht, Faculteit Farmaceutische Wetenschappen
PO BOX 80082, NL-3508 TB Utrecht

In the past few years there has been a growing interest in the use of Cannabis for medical purposes, e.g. treatment of nausea and vomiting in oncolytic therapy, multiple sclerosis, glaucoma and anorexia associated with AIDS [1].

This has led to the introduction of Medical Grade Marihuana (MGM©), of which name changed in 2000 to Medical Grade Cannabis (MGC©). A high level of product uniformity is reached by cloning a variety of *Cannabis sativa* L., and keeping growth conditions almost the same. These include nutrients and breeding soil constitution, temperature, air-humidity, duration and intensity of lighting and harvest time. The flowering tops are harvesting and the remaining leaves strip off. These flowering tops are dried and packaged in airtight bags, protected from light and humidity. The packaged MGC© is sterilised by gamma irradiation.
Analyses for the content uniformity and stability has been performed [2]. The main cannabinoids constituents are Δ^9-TetraHydroCannabinbol (Δ^9-THC) and its 2-carboxylic acid (Δ^9-THCA). The amount of THCA exceeds the amount of the other cannabinoids by far. The product contains not less than 10.0 % w/w THC (calculated as the total amount of THCA + THC) and not more than 1.0 % w/w Cannabinol (CBN) (end of shelf-life specification). MGC proved to be rather stable [2]. The product can be kept for two years at room temperature (15-25°C) or less.

Because THCA is not available as a standard, the batches for the quality controll are routineously assayed by means of TLC and HPLC before and after decarboxylising THCA by heating at 100 °C for 2.5 hours. The mathematics calculations for quantification is performed using the area under curve of the HPLC-graph, using the equations obtained from the calibration curves.

Further analytical specifications such as limits on the microbiological contamination, pesticide residues, heavy metals proved to be well in range of the demands on vegetable drugs in Ph. Eur. **2002** [3].

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CANNABINOIDS EFFICACY IN REDUCTION OF INTRAOCULAR PRESSURE

Anna-Maria Szczesniak, Melanie Kelly, and Orlando Hung
Dalhousie University, Department of Pharmacology, Halifax,
Nova Scotia, B3H 4H7, Canada

Cannabinoids have been suggested to produce beneficial therapeutic hypotensive effects in the management of patients with glaucoma, a disease in which elevated intraocular pressure (IOP) is a primary risk factor. Although a number of studies have reported that cannabinoid compounds can decrease IOP, the relative efficacies of these compounds in lowering IOP or their cellular mechanisms of actions were not evaluated.

The objective of our study was to compare the pharmacodynamic effects (following intraperitoneal administration) of a liposome-encapsulated Δ^9-tetrahydrocannabinol (LTHC) preparation on IOP in Brown Norway rats, and to compare this to the effects of two synthetic cannabinoid receptor agonists, Methanandamide (MA) and WIN55, 212-2.

IOP was measured with the use of a tonometer (Tono-Pen®XL). The animals were placed on a counter and the tonometer was placed gently against the anesthetized surface of the cornea. For each time point, IOP was measured ten times and the mean value was calculated. Drugs were administered via IP injection, and IOP readings were taken every 15 min for the period of 2 hrs.

The administration of LTHC, WIN55, 212-2, and MA reduced IOP in rat eyes. A reduction from 20 ±0.5 to 17.5±0.5 was seen at 30-60 min after administration of WIN55, 212-2 and MA. The administration of LTHC, also produced a decrease in IOP (20±0.5 to 18.5±0.5), however to lesser degree than the two synthetic agonists. The IOP lowering effect of LTHC was significantly reduced by IP administration of AM251, a CB_1 cannabinoid receptor antagonist.

RT-PCR, using primers specific for the rat CB_1 receptor, confirmed the expression of mRNA for CB_1 receptors in the ciliary body and retina of rat eye. A PCR product of 520 bp was obtained. The PCR product was cloned and sequenced and found to correspond with the CB_1 receptor sequence in GenBank (Accession # 004350). Western Blot analysis confirmed the presence of the 53 kDa CB_1 receptor protein.

In conclusion, the administration of 75ug of MA and 0.5 mg of WIN55, 212-2 produced a greater reduction in IOP in Brown Norway rats than the administration of 60 or 120 ug of LTHC. The presence of CB_1 receptors in the ciliary body, suggests that the effects of cannabinoid agonists on IOP may be mediated, in part, via these receptors to produce alterations in aqueous humor formation.

THE CB_1 RECEPTOR ANTAGONIST SR141716A SELECTIVELY INCREASES MONOAMINERGIC NEUROTRANSMISSION IN THE MEDIAL PREFRONTAL CORTEX: IMPLICATIONS FOR THERAPEUTIC ACTIONS

Eleni T. Tzavara, Richard J. Davis, Kenneth W. Perry, Craig Salhoff,
Frank P. Bymaster, and George G. Nomikos
Eli Lilly and Company, Lilly Corporate Center, Neuroscience Discovery Research, Indianapolis, IN 46285-0510

Cannabinoid antagonists seem to have a promising therapeutic potential in a number of CNS related disorders. Clinical trials have shown SR141716A, the potent and selective prototypical inhibitor of the CB_1 receptors, to be effective in the treatment of obesity. Studies in animal models suggest possible antipsychotic- and antidepressant-like actions, memory enhancing properties and a role in the treatment of addiction for this class of compounds. At doses similar to those effective in behavioral tests, we, and others have shown an excitatory action of SR141716A in neurotransmitter release in the anterior hypothalamus and the hippocampus. Here, we have focused on the effects of acute administration of SR141716A on monoamine efflux in the medial prefrontal cortex (mPFC) versus the nucleus accumbens (nAcc). In vivo microdialysis studies were performed according to the guidelines of the Animal Care and Use Committee of Eli Lilly and Co. Male Wistar rats (250-300 g) were implanted with concentric microdialysis probes in the medial prefrontal cortex (BAS, BR-4) or in the n.Acc (BAS, BR-2). About 24 hr later, a modified Ringer's solution was perfused at a rate of 1 μl/min, samples were collected every 30 min. and analyzed the same day of the experiment with HPLC coupled to electrochemical detection. SR141716A 3mg/kg and 10mg/kg (dissolved in 2%DMSO, 2% cremophor EL, saline) or vehicle were injected i.p. at a volume of 3ml/kg. For PCP-induced hyperlocomotion, SR141716A was administered simultaneously with PCP (3mg/kg) and locomotor activity was monitored for 60 min. I) In agreement with previous studies, SR141716A increased acetylcholine (ACh) efflux in the mPFC, in a dose dependent manner. On the contrary, it had no effect on ACh in the nAcc. II) SR141716A moderately increased serotonin efflux and contents of its metabolite 5-HIAA, both in the mPFC and the nAcc, and increased norepinephrine (NE) and dopamine (DA) and their metabolites in the mPFC. Interestingly, it had no effect on NE, DA and their metabolites in the nAcc. III) At the same doses, SR141716A dramatically attenuated PCP-induced hyperlocomotion in mice. Thus, psychostimulant compounds, but apparently not SR141716A, increase DA and decrease ACh efflux in the nAcc. On the contrary, SR141716A increases cortical DA, which has been shown to suppress DA activity in the nAcc, in line with its proposed anti-addictive properties. In addition, SR141716A stimulates cholinergic activity in the cortex, and increases DA selectively in the mPFC versus the nAcc, a hallmark of action of atypical antipsychotics. Efficacy of this drug in the PCP-induced hyperlocomotion paradigm, further supports antipsychotic activity of cannabinoid antagonists. Antipsychotic-like and procholinergic (a purported mechanism of action of drugs that improve cognition) properties of cannabinoid antagonists together with their mild stimulatory effects on serotonin and NE efflux makes such compounds unique candidates in the treatment of psychosis and affective disorders.

PROCONVULSIVE AND NEUROTOXIC EFFECTS OF ANANDAMIDE IN MICE LACKING FATTY ACID AMIDE HYDROLASE

Angela B. Clement, E. Gregory Hawkins, and Benjamin F. Cravatt
The Skaggs Institute for Chemical Biology and Departments of Cell Biology and Chemistry, The Scripps Research Institute, CA 92037, USA

Studies directed towards potential neuroprotective or neurotoxic properties of the putative endocannabinoid anandamide have so far shown ambiguous results. The major problem studying anandamide function is the rapid degradation of anandamide to arachidonic acid and ethanolamine by fatty acid amide hydrolase (FAAH), with arachidonic acid itself exhibiting a variety of biological effects. Here we used mice lacking FAAH [FAAH (-/-)] to study anandamide effects in models of chemically induced epileptic seizures. We show that FAAH (-/-) mice are more sensitive to kainate - induced seizures than FAAH (+/+) mice probably due to the higher anandamide content in the brain. When injected with anandamide alone, only FAAH (-/-) mice show dose-dependent seizure behavior. In addition, anandamide treatment enhances seizure susceptibility of FAAH (-/-) mice to bicuculline and kainate. The enhanced seizure susceptibility elicited by exogenous administered anandamide in the bicuculline and kainate model correlates with neuronal injury in the CA1 and CA3 regions of the hippocampus, respectively. The proconvulsive and neurotoxic anandamide effects are reversed by low doses of the CB_1 receptor antagonist SR141716A (1 -3 mg/kg i.p.) in the bicuculline model. Since SR141716A itself strongly enhanced kainate-induced seizures in FAAH (-/-) mice at low doses (≥ 1 mg/kg, i.p.), attempts to block the anandamide effect in the kainate model failed. Collectively, our results demonstrate clear proconvulsive and neurotoxic effects of anandamide and indicate the involvement of CB_1 receptor signaling.

RESIDUAL (NON-ACUTE) NEUROCOGNITIVE CONSEQUENCES OF MARIJUANA: A META-ANALYTIC STUDY

Igor Grant, Raul González, Catherine Carey, Loki Natarajan, and Tanya Wolfson
Department of Psychiatry, University of California, Center for Medicinal Cannabis Research, San Diego, California 92103

The possibility that cannabinoids might be used medicinally for chronic diseases emphasizes the need to understand the potential long-term central nervous system consequences of these compounds. To provide a quantitative synthesis of research investigating the non-acute (residual) effects of marijuana use on neurocognitive performance of long-term users, two reviewers conducted independent literature searches and arrived at a consensus of 1,014 articles, 39 of which were ultimately selected for coding. The studies selected were scientific investigations with adult human subjects examining the effects of non-acute marijuana use on neurocognitive functioning that met essential a priori inclusion criteria. Ten studies met all of the original inclusion criteria, providing data for a total of 521 cannabis users and 376 non- or minimal users. Neuropsychological results were grouped into eight ability domains, and effect sizes were calculated by domain for each study individually, and combined for the full set of studies. Using slightly liberalized criteria, an additional 5 studies were included in a second analysis, bringing the total number of subjects to 1,053 (i.e., 602 cannabis users and 451 non-users). Effect sizes for domains (with one exception) and the overall effect size across domains included zero, suggesting a lack of effect. A very small effect was observed for the learning domain. There are few studies on the chronic, long-term CNS effects of cannabis that meet current research standards. The studies that met our criteria yielded no basis for concluding that long-term cannabis consumption is associated with generalized neurocognitive decline, with the possible exception of slight decrements in the area of learning new information.

THE EFFECT OF CANNABINOIDS ON SENSORIMOTOR GATING IN SWISS MICE

Daniel Malone, Leonora Long, Larissa Gampert, and David Taylor
Department of Pharmaceutical Biology and Pharmacology,
Monash University, 381 Royal Parade, Parkville, AUSTRALIA

Whist a number of clinical studies have described psychotic symptoms in people after cannabis ingestion, it is still unclear whether cannabis consumption directly causes psychosis or whether it precipitates psychosis in individuals with existing abnormal neurochemistry. Prepulse inhibition (PPI) of the startle reflex is an operational measure of sensorimotor gating, and is impaired or disrupted in schizophrenia patients and in rodents treated with psychotomimetic drugs such as amphetamine. This project aimed to investigate the acute effects of the CB_1 receptor agonist Δ^9-tetrahydrocannabinol (Δ^9-THC) and the CB^1 receptor antagonist/inverse agonist N-piperidino-5-(4-chlorphenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide (SR141716) on PPI in Swiss mice. Experiments were also performed with the above cannabinoids co-administered with apomorphine, a D1/D2 receptor agonist known to disrupt PPI. Following drug administration, mice were placed in SR-LAB startle chambers (background noise level of 70 dB) which measured startle reactivity to acoustic trials. Mice were subjected to acoustic trials which consisted of either a single pulse of 120 dB and 40 ms duration (PULSE), a single prepulse of 20 ms duration that was 3, 6 or 12 dB above background (PREPULSE), a PULSE preceded 100 ms by a PREPULSE (PREPULSE + PULSE) or the background alone. The whole body startle response elicited by each acoustic trial was detected by a piezoelectric accelerometer attached to the mouse enclosure. PPI was calculated using the following formula: %PPI = 100 -100 x (PREPULSE + PULSE)/(PULSE). Δ^9-THC (0.3, 1.0 and 3.0 mg/kg i.v.) alone had no significant effect on PPI, whilst both doses of SR141716 used (1 and 3 mg/kg i.p.) significantly increased PPI. Apomorphine (0.3, 1.0 and 3.0 mg/kg i.p.) significantly disrupted PPI at the highest dose tested. When apomorphine 1 mg/kg i.p. was administered before Δ^9-THC, there was no significant effect on PPI compared with controls. When apomorphine 3 mg/kg i.p. was administered before Δ^9-THC, there was a significantly greater disruption produced than that of apomorphine alone. These results suggest that Δ^9-THC does not affect PPI alone, but when sufficient dopaminergic stimulation is induced by apomorphine, Δ9-THC enhances disruption of PPI, indicative of altered sensorimotor gating. This implies that an individual with a compromised dopaminergic function may be at greater risk of developing psychosis after the consumption of cannabis. However, as the actions of cannabis in the central nervous system are complex, the ability of cannabinoids to alter neurotransmitter systems in order to produce disruption in sensorimotor gating requires further investigation.

DISRUPTION OF A549 PULMONARY EPTIHELIAL CELL ENERGETICS BY Δ^9-TETRAHYDROCANNABINOL

Theodore A. Sarafian, Shahig Kouyomjian, Farnaz Khoshagideh,
Donald P. Tashkin, and Michael D. Roth
Department of Medicine, Division of Pulmonary and Critical Care, UCLA,
Los Angeles, California 90095, USA

Δ^9-tetrahydrocannabinol (THC) suppresses anti-fas-induced apoptosis while promoting necrotic death in the adenocarcinoma cell line A549. In order to investigate mechanisms underlying this effect we examined mitochondrial function in THC-treated cells. Using a firefly luciferase assay, we observed that 24 hr THC exposure resulted in a concentration-dependent decrease in ATP level with an IC_{50} of ~5 μg/ml. Flow cytometry studies using a probe for mitochondrial membrane potential, JC-1, revealed a dramatic decrease in red fluorescence caused by 24 hr exposure to 5 μg/ml THC. This decrease was concentration-dependent (IC_{50} ~ 1-2 μg/ml) and attenuated by cyclosporin A, suggesting a role for the mitochondrial permeability transition pore. Treatment with carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP) caused a similar decrease in red fluorescence, whereas oligomycin increased red fluorescence, suggesting that THC acted more like an electron transport uncoupler than an ATP synthase inhibitor. Exposure of cells to smoke particulate fraction at 50 μg/ml tar resulted in a mean JC-1 red fluorescence of 1662 for tobacco extract but only 293 for marijuana extract. The equivalent tar extract from a placebo marijuana cigarette devoid of THC produced a mean JC-1 red fluorescence of 1578. However, when purified THC (5 μg/ml) was added to this extract the mean red fluorescence decreased to 299. These results indicate that THC can decrease mitochondrial membrane potential in the presence of the complex mixture of the cigarette smoke particulate fraction and that THC is the major contributor to the disruption of mitochondrial membrane potential in A549 cells. The observed effects on pulmonary mitochondrial cell energetics may explain the reported THC-induced inhibition of apoptosis and identify new potential health risks associated with marijuana smoking.

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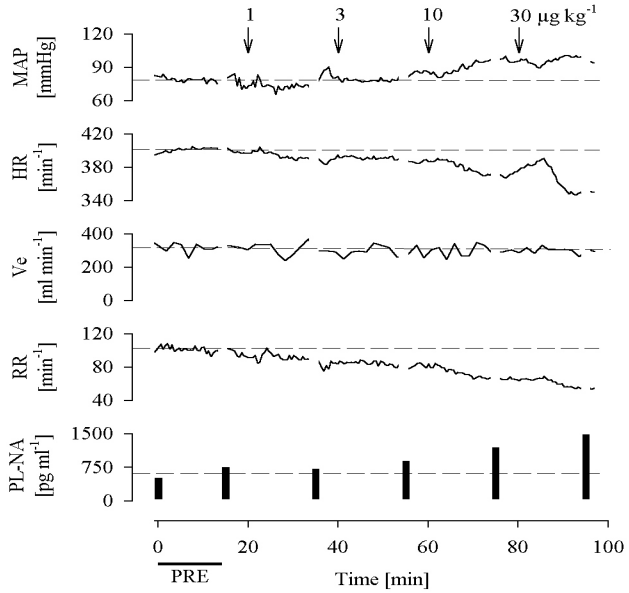
CENTRAL RESPIRATORY EFFECTS OF CANNABINOIDS IN RATS

Bela Szabo, Torsten Pfitzer, and Nathalie Niederhoffer
Institut für Pharmakologie, Albert-Ludwigs-Universität, D-79104 Freiburg, Germany

We previously showed that systemic administration of cannabinoids in anesthetized rats markedly reduces the respiratory rate through activation of CB_1 receptors. The objective of the present study was to determine whether cannabinoids induce respiratory depression by acting in the central nervous system.

Respiratory and cardiovascular responses to intracisternal injection (i.c.) of the mixed CB_1/CB_2 cannabinoid receptor agonist WIN55212-2 ((+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]1,4-benzoxazinyl]-(1-naphthalenyl) methanone mesylate) were evaluated in anesthetized rats. WIN55212-2 (1-30 µg kg^{-1}) caused dose-dependent respiratory changes (see figure): respiratory rate decreased (by 38±3 % after 30 µg kg^{-1}), tidal volume increased (by 24±4 % after 30 µg kg^{-1}) and minute respiratory volume decreased (by 23 % after 30 µg kg^{-1}). WIN55212-2 also dose-dependently decreased heart rate (by 10±3 % after 30 µg kg^{-1}) and increased the plasma noradrenaline concentration (by 145±36 % after 30 µg kg^{-1}); mean arterial pressure was significantly enhanced (by 20±6 % after 30 µg kg^{-1}). I.c. administration of WIN55212-3, the enantiomer of WIN55212-2 lacking affinity for cannabinoid receptors, had no effects on the measured parameters.



Respiratory and cardiovascular effects of WIN in an anesthetized rat.

After a 15-min baseline period (PRE), WIN was injected i.c. as indicated by the arrows. Mean arterial pressure (MAP), heart rate (HR), maximal airflow (Ve) and respiratory rate (RR) were monitored continuously; the plasma noradrenaline concentration (PL-NA) was measured twice during baseline and 15 min after each WIN dose. The original tracings represent 8 experiments with similar results.

The results show that injection of WIN55212-2 into the cisterna cerebellomedullaris of anesthetized rats decreases breathing frequency and minute respiratory volume, most probably through activation of specific cannabinoid receptors in the medulla oblongata. This centrally-mediated effect likely accounts for the marked respiratory depression observed after systemic administration of cannabinoid agonists. However, a direct action in the lung cannot be ruled out. Centrally administered cannabinoids also enhance sympathetic tone and cardiac vagal tone, leading to hypertension and bradycardia (as already demonstrated in rabbits; Niederhoffer and Szabo, J Pharmacol Exp Ther 294, 707-713, **2000**).

CHANGES IN ENDOCANNABINOID CONTENTS IN THE BRAIN OF RATS CHRONICALLY EXPOSED TO NICOTINE, ETHANOL OR COCAINE

Sara González, Maria Grazia Cascio[1], Javier Fernández-Ruiz, Filomena Fezza[1], Vincenzo Di Marzo[1], and José A. Ramos
Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Complutense, 28040-Madrid (Spain); [1]Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Pozzuoli, Napoli (Italy)

Despite recent data suggesting that the endocannabinoid transmission is a component of the brain reward system and plays a role in dependence/withdrawal to different habit-forming drugs, only a few studies have examined changes in endocannabinoid ligands and/or receptors in brain regions related to reinforcement processes after a chronic exposure to these drugs. Recently, we performed a comparative analysis of the changes in cannabinoid CB_1 receptor density in several rat brain regions caused by chronic exposure to some of the most powerful habit-forming drugs (see González et al., Drug Alcohol Depend. 66, 77-84, **2002**). In the present study, we have extended this objective by examining changes in the brain contents of arachidonoylethanolamide (AEA) and 2-arachidonoyl-glycerol (2-AG), the endogenous ligands for cannabinoid receptors, in animals chronically exposed to cocaine, nicotine or ethanol. Results were as follows. Cocaine was the drug exhibiting the minor number of effects, with only a small, but significant, decrease in the content of 2-AG in the limbic forebrain. By contrast, chronic alcohol exposure caused a decrease in the contents of both AEA and 2-AG in the midbrain, while it increased AEA content in the limbic forebrain. This latter effect was also observed after chronic nicotine exposure together with an increase in AEA and 2-AG contents in the brainstem. By contrast, the hippocampus, the striatum and the cerebral cortex exhibited a decrease of AEA and/or 2-AG contents after chronic nicotine exposure. We also tested the effect of chronic nicotine on brain CB_1 receptors, which had not been investigated before, and found an almost complete lack of changes in mRNA levels or binding capacity for these receptors. In summary, our results, in concordance with previous data on CB_1 receptors, indicate that the three drugs tested here produce different changes in endocannabinoid transmission. Only in the case of alcohol and nicotine, we observed a common increase of AEA contents in the limbic forebrain. This observation is important considering that this region is a key area for the reinforcing properties of habit-forming drugs, which might support the involvement of endocannabinoid transmission in some specific events of the reward system activated by these drugs.

EFFECTS OF A PAVLOVIAN CONDITIONING PARADIGM ON THE DEVELOPMENT OF CANNABINOID TOLERANCE

Matthew Hill, Boris Gorzalka, and Joyce Choi
Department of Psychology, University of British Columbia,
Vancouver, B.C., V6T1Z4, Canada

The development of tolerance to cannabinoid administration has been observed in humans and animals, but research on the influence of conditional factors has been contradictory. The purpose of this investigation was to examine the effects, in female rats, of a standard Pavlovian conditioning paradigm on the development of tolerance to hypolocomotion induced by the potent cannabinoid agonist HU-210. One group of rats was administered drug and placebo in a conditional fashion with drug in the CS+ environment and placebo in the CS-. The second group of rats was administered the drug and placebo in a counterbalanced, pseudo-random fashion in both environments to prevent association of drug administration with contextual cues. The results indicate that rats in the associative paradigm developed tolerance significantly faster than those in the non-associative group. On the test day, in which the conditional group received drug and placebo in the opposite environments, it was found that drug in the CS- environment did not result in behavioural relapse, but that placebo in the CS+ environment resulted in a trend of increased activity levels. Taken together, these findings suggest that the development of tolerance to hypolocomotion is augmented by the presence of predictive environmental cues, and furthermore that the development of tolerance is not reversible by administering the drug in a novel environmental setting. This suggests that tolerance is ultimately guided by physiological alterations in cannabinoid receptor density and endogenous ligand availability, and that the development of this tolerance is enhanced by predictive associations.

DISRUPTION OF CB_1 RECEPTOR GENE EXPRESSION ATTENUATES ALCOHOL-SEEKING BEHAVIOR IN MICE

Basalingappa L. Hungund[1,2,3], Istvan Szakall[2],
B. S. Basavarajappa[1,2], and Csaba Vadasz [2,4]
[1]NYS Psychiatric Institute, New York, NY 10032,USA
[2]Nathan Kline Institute, Orangeburg, Orangeburg, NY 10962, USA
[3]College of Physicians & Surgeons, Columbia University, New York, NY 10032, USA
[4]New York University, New York, NY

Recent evidence suggests that many of the actions of alcohol including alcohol –seeking behavior may be mediated through anandamide-cannabinoid (CB_1) receptor system (Basavarajappa et al. Brain Research 793: 212, **1998** and Basavarajappa et al. J. Neurochem 72: 522, **1999**). It has also been reported that pharmacological manipulation through the CB_1 receptor antagonist SR141716A reduced and CB_1 receptor agonist CP55,940 increased alcohol consumption in rodents. The current study examined the effect of targeted mutation of CB_1 receptor gene on alcohol-seeking behavior and we report here that mice lacking CB_1 receptors consumed significantly less alcohol and had lower preference for alcohol compared to their corresponding wild type counterparts.

Two groups of mice [a] CB+/+, [b] CB_1[-/-], n = 10 respectively (derived from heterozygous pairs provided by Dr. Andreas Zimmer), were subjected to alcohol preference test following the procedure for voluntary self administration (two bottle choice) developed by Vadasz et al. (Alcohol 22: 25, **2000**). Animals were acclimated to alcohol by offering escalating concentration of alcohol for a period of 72 h for each testing period beginning with a 3% solution for trial 1 (day 1-3) –was increased to 6% in trial 2 (days 1-3) and finally to four three-day trials each with 12% alcohol. The averages for alcohol, food and water consumption for each group were calculated from the final four three-day trials with 12% alcohol solution. The data for alcohol, water and food consumption are expressed as g/kg body wt/day. The values for alcohol preference is expressed as a ratio of alcohol consumed vs. total liquid intake.

No significant loss or gain in body weight was observed in the two groups during the entire testing period. Similarly, the treatment procedure did not cause any significant shift either in the water or food consumption between wild type and CB_1 knockout animals. However, a significant reduction in alcohol intake (77%, $p < 0.005$) was seen in mice lacking CB_1 receptors. The results suggest that the disruption of CB_1 receptor gene results in reduced preference (70%) for alcohol compared to wild type animals. These results strongly support a role for cannabinoid receptor system in alcohol-seeking behavior.

ETHANOL PREFERENCE AND ADDICTION IN CANNABINOID CB_1 RECEPTOR DEFICIENT MICE

Ildiko Racz, Andras Bilkei-Gorzo, Kerstin Michel, and Andreas Zimmer
Laboratory of Molecular Neurobiology, Department of Psychiatry, University of Bonn,
Sigmund-Freud-Str. 25, 53105 Bonn, Germany

Ethanol is thought to affect neurotransmission through differential interactions with membrane proteins, including neurotransmitter receptors. This idea is supported by studies in animals and men, which have demonstrated that ethanol drinking behavior can be modulated through pharmacological disruption of certain neurotransmitter systems. These findings provide a rational basis for the pharmacotherapy of alcoholism. We have studied the role of the cannabinoid CB_1 receptor in ethanol preference and addiction using CB_1 receptor knockout mice. These experiments were performed on a congenic a C57BL/6J genetic background, because this strain is known to have a high preference for ethanol.

We will report the acute effects of a single intraperitoneal injection of ethanol on body temperature and motor coordination in wild type and mutant animals. Ethanol tolerance and withdrawal was determined after chronic alcohol administration. Previous studies have shown that the consumption of ethanol can be stimulated by cannabinoids, while the CB_1 receptor antagonist SR141716A has been reported to reduce alcohol intake. We have now further investigated the role of CB_1 receptors on ethanol preference using a two bottle choice paradigm. The results of these experiments demonstrate that ethanol preference is generally similar between wild type and mutant mice. However, ethanol consumption can be differentially influenced by certain experimental conditions.

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